

# **EVALUATION OF CO-AGGLUTINATION TEST IN THE EARLY DIAGNOSIS OF LEPTOSPIROSIS**

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# *Certificate*

Certified that the dissertation entitled  
**“EVALUATION OF CO-AGGLUTINATION TEST IN THE  
EARLY DIAGNOSIS OF LEPTOSPIROSIS”** is a bonafide work  
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## **DECLARATION**

*I declare that the dissertation entitled “**EVALUATION OF CO-AGGLUTINATION TEST IN THE EARLY DIAGNOSIS OF LEPTOSPIROSIS**” submitted by me for the degree of M.D. is the record work carried out by me during the period of Jan 2006 to May 2007 under the guidance of Dr. G. Sumathi, M.D., Ph.D., Professor of Microbiology, Institute of Microbiology, Madras Medical College, Chennai and has not formed the basis of any Degree, Diploma, Associateship, Fellowship titles in this or any other University or other similar institution of higher learning.*

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# INTRODUCTION

*“To wrest from nature, the secrets which have perplexed philosophers in all ages, to track to their sources, the causes of disease, to correlate the vast stress of knowledge, that they may be quickly available for the prevention and cure of disease - these are our ambitions”*

**-William Osler**

Leptospirosis is a zoonotic disease caused the spirochaete of the genus *Leptospirra*. This disease is endemic in many parts India and causes frequent outbreaks. Early diagnosis has got very important therapeutic prognostic significance and helps in controlling the outbreaks.

Leptospirosis is an acute generalised infectious disease characterised by extensive vasculitis predominantly affecting the liver and kidneys. Though it is a disease of wild mammals and rodents, humans are infected occasionally through direct or indirect contact. In India it is under reported due to lack of laboratory facilities.

The pathogenic leptospira belong to the interrogans complex and are classified into 25 serogroups and more than 280 serovars.

In India leptospirosis has been documented since 1930's. The environmental conditions, host range and reservoir pattern make the country more ideal for the prevalence of leptospirosis<sup>59</sup>.

### *Leptospirosis - A re-emerging disease*

Leptospirosis is now identified as one of the emerging infectious diseases exemplified by recent large outbreaks in Nicaragua, Brazil, India (WHO 2000),<sup>58</sup> South East Asia, the United States (CPC 1998) and most recently in several other countries as a result of the increase in zoonotic disease.

In the Landmark Institute of medicine report “Emerging infections: microbial threats to Health in the United States”. Leptospirosis was used as an example of an infection which had in the past caused significant morbidity in military personnel deployed in tropical areas (Smith H.L. et al, 2000)<sup>52</sup>.

The clinical manifestations are extremely protean varying from a mild anicteric illness characterised by fever, myalgia & conjunctival suffusion to a severe life threatening illness with jaundice, renal failure, pneumonia, meningitis, hemorrhagic shock with mortality ranging from 5-40%. These non-specific manifestations make the diagnosis of leptospirosis very difficult. Hence laboratory support is necessary for early confirmation of the clinical diagnosis and to help in the diagnosis of cases with atypical signs & symptoms of leptospirosis. Accurate diagnosis provides insight into the extent of public health problem in a community.<sup>18</sup>

The standard procedures for diagnosis of leptospirosis are demonstration of organism by dark field microscopy, isolation of organism by culture, detection of antibodies in sera by serological tests and by molecular methods such as PCR & RAPD. Animal inoculation can also be done using weanling animals.



- The conventional methods such as Dark field microscopy & culture to detect leptospirosis in clinical samples are either too unreliable or too slow to contribute to early diagnosis. Positive result by culture takes weeks or even months after inoculation into suitable culture medium.
- Serology becomes an important diagnostic tool from the moment antibodies appear in detectable quantities in blood. This is about one week after the onset of illness. Further more, a second serum sample is essential to follow the evolution of antibody titres. Early antibiotic treatment inhibits antibody response, limiting the efficacy of serological tests.
- Leptospira circulate in the blood of the patient until about the 10th day after the onset of symptoms. Hence rapid detection of such small numbers of leptospira in clinical samples is essential as leptospira can run a fulminant course and patients may die before the development of characteristic clinical manifestation of leptospirosis or the appearance of leptospira antibodies or both<sup>19</sup>.

With the introduction of molecular techniques like Polymerase Chain Reaction (PCR) in the year 1985, rapid detection of small numbers of leptospira in clinical samples has become practical due to the amplification of leptospiral DNA.

PCR has shown to be both sensitive and rapid. The disadvantage of PCR is that it is highly expensive & needs considerable expertise which is not available in many laboratories in our country.

Hence the need of the hour is a simple, rapid, inexpensive test and reliable test for the early diagnosis of leptospirosis which can be employed in small laboratories & field settings.

# REVIEW OF LITERATURE

## HISTORY:

The clinical syndrome of febrile illness and jaundice caused by leptospirosis was first recognised by Adolph Weil, a German physician in 1886. Goldsmith first used the term Weil's disease in 1887. In 1907 Stimson demonstrated by Silver staining the presence of clumps of spirochaetes in the kidney tubules of a patient who reportedly died of yellow fever. The spirochaetes had hooked ends and Stimson named them *Spirochaeta interrogans* because of their resemblance to question mark. Unfortunately this sentinel observation was overlooked for many years (Faine et al. S, 1994)<sup>19</sup>.

The etiology of leptospirosis was demonstrated independently in 1915 in Japan and Germany (Everard J.D. - 1996). In Japan, Inada & Ido detected both spirochaetes & specific antibodies against them in the blood of Japanese miners with infectious jaundice. In Germany two groups of physicians studied German soldiers afflicted by "French disease" in the trenches of Northeast France. Uhlenhuth and Fromme detected spirochaetes in the blood of guinea pigs inoculated with blood of infected soldiers (Faine S, et.al. 1994)<sup>19</sup>.

The first isolate made in 1917 from a patient in Japan with Jaundice and hemorrhagic manifestations was named as *Icterohaemorrhagiae*. Subsequently other members of serogroups were isolated in various places of the world from different animals reservoir hosts. Inado identified *Leptospira hebdomadis* carried by

the field mouse as the causative agent for the non-icteric syndrome the "7 day fever"<sup>19</sup>.

#### **ALTERNATIVE NAMES FOR LEPTOSPIROSIS:**

Autumnal fever, Cane field fever, Canicola fever, Field fever, Hemorrhagic jaundice, Mud Fever, Rice-Field Fever, Seven Day Fever, Bushy-Creek Fever, Cane Cutter Fever, European Swamp Fever, Fort bragg Fever, Pea Pickers Disease, Spirochaetosis, Stuttgart Disease, Swamp Fever, Swineherd Disease, Wycon Fever & Weil's Disease.

#### **ETIOLOGY:**

Leptospire are spirochaetes belonging to the order Spirochaetales and family *Leptospiraceae*.

#### **CLASSIFICATION:**

The classification and the nomenclature of leptospira are complex but at the moment two different classifications are being used.

- I. Serological Classification (Based on Antigenic determinants)
- II. Genotypic classification (Based on genetic criteria)

#### **SEROLOGIC CLASSIFICATION:**

Prior to 1989, the genus *Leptospira* was divided into two species, *L.interrogans*, comprising of all pathogenic strains and *L. biflexa* containing the saprophytic strains isolated from the environment. (Faine Set al., 1982, Johnson R.C. et al, 1984). *L. biflexa* was differentiated from *L. interrogans* by the growth of the

former at 13°C and growth in the presence of 8-azaguanine (225 µg/ml) and by the failure of *L.biflexa* to form spherical cells in 1 m NaCl<sup>19</sup>.

Both *L. interrogans* & *L.biflexa* are divided into numerous serovars defined by cross-absorption with homologous antigen (Dikken H et al., 1978)<sup>13</sup>. If more than 10% of the homologous titer remains in atleast one of the two antisera on repeated testing, then the two strains are said to belong to different serovars International committee on systematic bacteriology & sub-committee on the taxonomy of *Leptospira*, 1987)<sup>27</sup>. Over 60 serovars of *L.biflexa* & over 220 serovars of *L.interrogans* have been recorded so far. Serovars that are antigenically related have traditionally been grouped into serogroups. While serogroups have no taxonomic standing, they have been proved to be useful for epidemiological purposes.

The serogroups of *L.interrogans* and some common serovars are shown in the following table.

**SEROGROUP & SEROVARs OF *L. INTERROGANS*<sup>20</sup>:**

<b>Serogroup</b>	<b>Serovars</b>
Icterohaemorrhagiae	<i>ictero haemorrhagiae, copenhageni, zimbabwe, lai</i>
Hebdomadis	<i>hebdomadis, jules, kremastos</i>
Autumnalis	<i>autumnalis, fortbragg, bim, weerasinghae</i>
Pyrogenes	<i>pyrogenes</i>
Bataviae	<i>bataviae</i>
Grippotyphosa	<i>grippotyphosa, canalzonae, ratnapura</i>

Canicola	<i>canicola</i>
Australis	<i>australis, bratislava</i>
Javanica	<i>javanica</i>
Sejroe	<i>sejroe, hardjo, saxkoebing</i>
Panama	<i>panama, mangus</i>
Cynopteri	<i>cynopteri</i>
Djasiman	<i>djasiman</i>
Sarmin	<i>sarmin</i>
Mini	<i>mini, georgia</i>
Tarassovi	<i>tarassovi</i>
Ballum	<i>ballum, aroborea</i>
Celledoni	<i>celledoni</i>
Louisiana	<i>louisiana, lanka</i>
Ranarum	<i>ranarum</i>
Manhao	<i>manhao</i>
Shermani	<i>shermani</i>
Hurst bridge	<i>hurst bridge</i>

#### **GENOTYPIC CLASSIFICATION:**

The phenotypic classification has been replaced by genotypic classification in which all the serovars are grouped under a number of genomospecies. Genetic heterogeneity of leptospira has been demonstrated long time back by Brendle (Brendle J.J. et al, 1974)<sup>7</sup>. DNA hybridisation studies has led to the recognition of 10

genomospecies of *Leptospira* (Yasuda P.H. et al, 1987)<sup>61</sup>. An additional genomospecies, *L.Kirschneri*, was added later (Ramadass P. et al., 1992)<sup>43</sup>. After an extensive study of several hundred strains, workers at the Center for Disease Control move recently defined 16 genomospecies of *Leptospira* that included those described previously and also five (5) new genomospecies (Brenner D.J., 1999)<sup>8</sup>. One of the five newly described was named *L.alexanderi*. An additional species *L.Fainei* has since been described, which contains a new serovar, *hurst bridge* (Perolat et al., 1998)<sup>35</sup>. The genotypic classification of leptospires is supported by multilocus enzyme electrophoresis data but recent studies suggest that further taxonomic revisions would likely to take place in future (Postic. D et al., 2000)<sup>36</sup>.

The genomospecies of *Leptospira* do not correspond to the previous two species (*L.interrogans* & *L.biflexa*) and indeed, pathogenic and non-pathogenic serovars occur within the same species.

#### **GENOMOSPECIES OF LEPTOSPIRA & SEROGROUPS (BRENNER ET AL PERELAT ET AL)<sup>8</sup>**

<b>SPECIES</b>	<b>SEROGROUPS</b>
L.interrogans	Icterohaemohagiae, Canicola, Pomona, Australis, Autumnalis, Pyrogenes, Grippotyphosa, Sejroe Djasiman, Hebdomadis, Bataviae Ranarum, Louisiana, Mini, Sarmin
L.noguchii	Panama, Automnalis, Pyrogenes, Louisiana, Bataviae, Tarassovi, Australis, Shermani, Djasiman Pomona

L.santarosai	Shermani, Hebdomadis, Tarassovi Pyrogenes, Autumnalis, Bataviae Mini, Grippotyphosa sejroe, Pomona ,Javanica, Sarmin, Cynopteni
L. Meyeri	Ranarum, Semaranga, Sejroe, Mini Jarvanica
L wolbachii	Codice
L biflexa	Semaranga, Andamana
L.fainei	Hurst bridge
L. borgpetersenii	Javanica, Ballum, Hebdomadis Sejroe, Tarassovi, Mini, celledoni Pyrogenes, Bataviae, Australis, Autumnalis
L.weilii	Celledoni, Icterohaemorrhagiae Sarmini, Jarvanica, Mini, Tarassovi, Hebdomadis, Pyrogenes, Manhas, Sejroe
L. kirschneri	Grippotyphosa, Autumnalis, Cynopteri, Hebdomadis, Australis Pomona, Djasiman, Canicola, Icterohaemorrhagiae, Bataviae
L. inadia	Lyme, shermani, Tarassovi, Manchao, Canicola, Panama, Javanica
L.parva	Turneria
L.alexanderi	Manhao, Hebdomadis, Javanica, Mini
a. Serogroups	Semaranga, Andamana, codice, Turneria contain non-pathogenic leptospire
b.	Non-pathogenic strains of these species are known

Neither serogroup nor serovar reliably predicts the species of leptospira.

## **MORPHOLOGY**

Leptospires are tightly coiled spirochaetes, usually  $0.1\mu\text{m} \times 0.1\text{-}6\text{-}20\mu\text{m}$  but occasional cultures may contain much longer cells. The helical amplitude is approximately  $0.10$  to  $0.15\ \mu\text{m}$  and the wavelength is approximately  $0.5\ \mu\text{m}$  (Faine S et al, 1999)<sup>20</sup>. The cells have pointed ends, either or both of which are usually bent into a distinctive hook. Two axial filaments (periplasmic flagella) with polar insertions are located in the periplasmic space. The structure of the flagellar proteins is complex (Swain R.H.A., 1957)<sup>55</sup>.

Leptospires exhibit two distinct forms of movement, translational and non-translational. Morphologically all leptospires are indistinguishable, but the morphology of individual isolates varies with subculture in vitro and can be restored by passage in hamsters. (Ellis W.A. et al, 1982)<sup>16</sup>.

Leptospires have a typical double membrane structure in common with other spirochaetes in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlain by an outer membrane. Leptospiral lipopolysaccharide has a composition similar to that of other gram negative bacteria but has lower endotoxic activity (Shimizu T. et al., 1987)<sup>50</sup>.

Though there are more than 280 serovars of leptospira many of them are cross-reactive due to the overlapping of the antigenic structure. Two types of antigen have been identified. The surface antigen contains protein polysaccharide and is serovar specific,



while the somatic antigen contains genus specific lipopolysaccharide (S. Faine, 1982)<sup>18</sup>. The outer membrane of the organism is a potent immunogen and is the target for the immunoglobulin - complement mediated bactericidal action.

Leptospira possesses a thermolabile oxygen stable, soluble hemolysin and various other enzymes like catalase, lipase, oxidase, peroxidase and hyaluronidase. Presence of endotoxin has also been shown in certain serovars. Neither bacteriophages nor plasmids have been isolated from leptospires (S. Faine, 1999)<sup>20</sup>.

## **CULTURE METHODS**

Leptospires are obligate aerobes with an optimum growth temperature of 28<sup>0</sup>C to 30<sup>0</sup>C. They grow in simple media enriched with vitamins (Vit B<sub>2</sub> & B<sub>12</sub> growth factors), long chain, fatty acids and ammonium salts. Long chain fatty acids are utilised as the sole carbon source and are metabolised by  $\beta$ -oxidation (Johnson R.C. et al., 1984)<sup>31</sup>.

Growth of leptospires in media containing serum or albumin plus polysorbate and in protein-free synthetic media has been described (Turner L.H., 1970)<sup>56</sup>. Several liquid media containing rabbit serum were described by Fletcher, Korthoff, Noguchi and Stuart (Turner L.H., 1970)<sup>56</sup>. The most widely used medium in current practice is based on the oleic acid albumin medium EMJH (Ellinghausen H.C. et al., 1965,<sup>15</sup> Johnson R.C. et al., 1976)<sup>31</sup>. This medium is commercially available and contains tween 80 and bovine serum albumin. Some strains are more fastidious and require the addition of either pyruvate (Johnson R.C. et al., 1976)<sup>31</sup> or rabbit serum (Ellis W.H. et al., 1976)<sup>16</sup> for initial isolation. Growth

of contaminants from clinical specimens can be inhibited by the addition of 5-Fluorouracil (Johnson R.C. et al., 1976)<sup>31</sup>. Protein free media have been developed for use in vaccine production (Bey R.F. et al., 1978)<sup>6</sup>.

Growth of leptospires is often slow on primary isolation and cultures are retained for upto 13 weeks before being discarded, but pure subcultures in liquid media usually growth within 10 to 14 days. In semi solid media (Agar 0.1 - 0.2%) growth reaches a maximum density in a discrete zone beneath the surface of the medium, which becomes increasingly turbid as incubation proceeds. This growth is related to the optimum oxygen tension (Faine S et al., 1999)<sup>20</sup> and is known as Dinger's ring (Dinger J.E. et al., 1932)<sup>12</sup>. Leptospiral cultures may be maintained by repeated subculture or preferably by storage in semisolid agar containing haemoglobin (Faine S et al., 1999)<sup>20</sup>. Long term storage can also be done by lyophilisation or by storing at -70°C (Alexander, A.D. et al., 1972.<sup>2</sup>

#### **MOLECULAR BIOLOGY:**

Leptospires are phylogenetically related to other spirochaetes. The leptospiral genome is approximately 5,000 kb in size although smaller estimates have been reported. The genome is comprised of two sections, a 4,400 kb chromosome and a smaller 350 kb chromosome (Zuerner R.L. 1991)<sup>63</sup>. Other plasmids have not been reported. Leptospires contain two sets of 16S and 23s rRNA genes but only one 5S rRNA gene (Fukunaga M., et al., 1989)<sup>24</sup> and the rRNA genes are widely spaced.

The study of leptospiral genetics has been slowed by the lack of a transformation system. Recently, a shuttle vector was

developed using the temperature bacteriophage LE1 from *L. biflexa* (Saint Girons. I et al., 2000)<sup>48</sup>. This advance offers the prospect of more rapid progress in the understanding of leptospira at the molecular level.

Several repetitive elements have been identified (Zuerner, R.L. et al., 1991)<sup>63</sup> of which several are insertion sequences (IS) coding for transposases.

A number of leptospiral genes have been cloned and analysed including those for amino acid synthesis (Zuerner R.I et al., 1991)<sup>63</sup> rRNA ribosomal proteins, RNA polymerase, DNA repair, hemolysins, outer membrane proteins, flagellar proteins lipopolysaccharide synthesis.

## **PATHOGENESIS**

Once leptospira gain entry in human body they spread through the blood stream to all organs. Avirulent strains fail to multiply in the body and are removed from the blood in a day or two. Virulent leptospines multiply in the blood stream and in various organs and body fluids including the CSF. The organisms at this stage can be recovered from all tissues (leptospiremic stage) for 4-7 days. Agglutinating antibodies start appearing in the blood around 5<sup>th</sup> day (immune stage). (Faine S et.al. 1999)<sup>20</sup>. The organisms are then opsonised and removed from the blood by reticulo-endothelial system. These agglutinating antibodies (IgM>IgG) are detectable by the MAT (Microscopic agglutination test).

After 4-7 days, the organisms persist in the aqueous humor of the eye, the renal tubules and are excreted in the urine. The leptospiruria persists for 1-4 weeks in man.

Three mechanisms are involved in the pathogenesis of tissue injury in leptospirosis<sup>20</sup>.

1. Direct effect of leptospirosis
2. Immunological reaction
3. Non-specific factors.

#### **DIRECT EFFECT OF LEPTOSPIRES**

Leptospire lead to extensive endothelial injury resulting in multiple haemorrhages throughout the body transudation of fluid from the vascular compartment and hypovolemia. The peptidoglycan component of its cell wall induces monocyte to secrete TNF- $\alpha$  (tumor-necrosis factor- $\alpha$ ) and other cytokines to cause tissue injury. The endotoxin from leptospira is another major mediator of cell injury. In the kidney, leptospire take the following route: Glomeruli ==> Tubular lumen ==> Tubular necrosis and Acute interstitial nephritis. These lesions are probably caused by the leptospiral endotoxins.

In the liver, ultrastructural and histochemical studies strongly suggest that the fundamental lesion is at the subcellular level affecting vital systems. In human leptospirosis, severe pathological lesions occur at a time when leptospire are difficult to demonstrate in the liver, leading to the view that leptospiral toxins or release of cytokines may be the immediate cause of liver injury (S. Faine, 1999)<sup>20</sup>.

## IMMUNOLOGICAL REACTION:

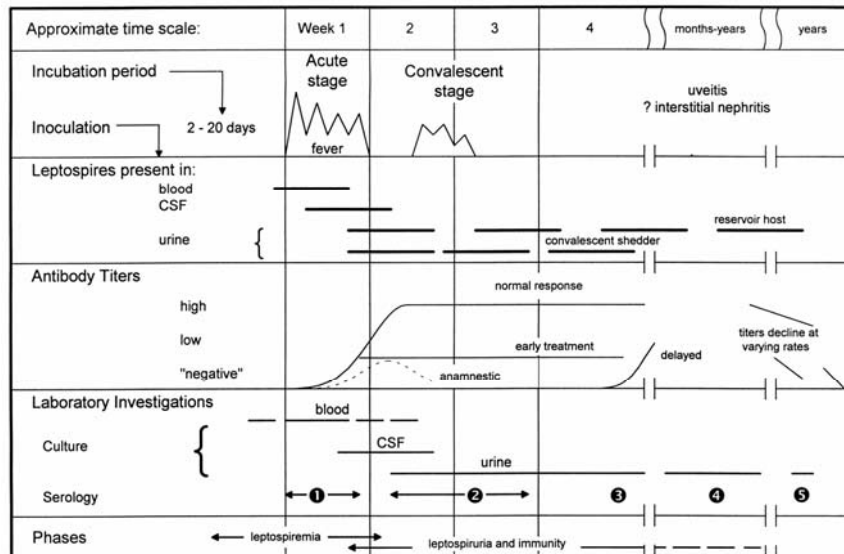
Meningeal inflammation and uveitis in leptospirosis are temporarily related to the rising titre of antibodies and occur during the immune phase. Immunological reactions probably account for the meningeal & uveal lesions (Faine S et al., 1999)<sup>20</sup>.

## NON SPECIFIC FACTORS:

Hypovolemia, hyperviscosity, intravascular coagulation, myocardial dysfunction, intravascular hemolysis and myoglobinuria have all been reported in patients with leptospirosis. These factors combine to produce widespread disturbances in the microcirculation and hemodynamic abnormalities. (Avean V.M., 1962)<sup>4</sup>.

## CLINICAL FEATURES:

Leptospirosis is an acute febrile generalised disease with a varied clinical picture ranging from a mild insignificant type to the florid fulminant septicaemic type with intermediate types of clinical disease in between. The incubation period is variable between 3-30 days with mean period of 8-12 days (Faine S et al., 1999)<sup>20</sup>.



**Biphasic nature of leptospirosis and relevant investigations at different stages of disease.** Specimens 1 and 2 for serology are acute-phase specimens, 3 is a convalescent-phase sample which may facilitate detection of a delayed immune response, and 4 and 5 are follow-up samples which can provide epidemiological information, such as the presumptive infecting serogroup. **(Courtesy: Turner L.H, 1970)** <sup>56</sup>

The main presenting symptoms are sudden onset of headache, muscle pain and muscle tenderness, fever with or without rigors, nausea vomiting, conjunctival suffusion, a transient skin or mucosal rash and signs of meningism.

The subsequent course depends on the type of infecting serovar, the number of infecting leptospires and the immune status of the patient. 5-10% of those infected with serovars like *icterohaemorrhagiae*, *copenhageni*, *bataviae* have been reported to develop Weil's disease syndrome. Infections with other serovars which cause milder illness are almost never icteric or fatal (Faine et.al 1999) <sup>20</sup>.

ARDS (Acute respiratory distress syndrome) and hemoptysis are common in infection with some serovars like *icterohaemorrhagiae* and *betaviae* (P, Merien F et al.2000) <sup>41</sup>.

These initial symptoms are co-incident with the septicaemic or leptospiraemic phase when leptospires circulate in the blood, urine and CSF. In mild forms the temperature subsides and recovery of all organs and functions is usually complete within 3-6 weeks after the onset of the disease.

In more severe forms, the initial illness is prolonged and diphasic. The fever subsides after 4-7 days to recur after 1-3 days of apparent recovery. This tissue or immune phase is characterised by saddle shaped fever temperature chart showing temperatures as high as 40<sup>0</sup>C and accompanied by more severe generalised manifestations and worsening prostration. The distinction between these two phases is often unclear and they merge in to a single continuous phase. In this phase more severe muscle pain especially of the calf muscle may occur (Rhabdomyolysis).

## **EPIDEMIOLOGY**

### *Source of infection*

Leptospirosis is presumed to be the most widespread zoonosis in the World (WHO report, 1999)<sup>58</sup>. The source of infection in humans is usually by direct or indirect contact with infected animals. Leptospire are parasites of both wild & domestic animals. In developing countries there are greater opportunities for exposure of the human population to infected animals whether live stock, domestic pets or wild and feral animals. Infected animals can be divided into maintenance hosts and accidental hosts. The disease is maintained in nature by chronic infection of the renal tubules of maintenance hosts (Faine S. et al, 1999)<sup>20</sup>.

The most important maintenance hosts are rodents which may transfer the infection to domestic farm animals, dogs and humans. The extent to which the infection is transmitted depends on many factors including climate population density and the degree of contact between maintenance hosts and human population. Different rodent species may be reservoirs of distinct serovars but rats are generally maintenance hosts for serovars of

the serogroup *Icterohaemorrhagiae*. Mice are maintenance hosts for the serogroup *Ballum*, Domestic animals also act as maintenance hosts, dairy cattle may harbour serovars *hardjo*, *grippityphosa*, pigs may harbour serovar *Pomona*, *tarassovi*, & *bratislava*; sheep may harbour serovars *hardjo* & *pomona* and dogs may harbour serovar *canicola*. (Faine et. al 1999)<sup>20</sup>

Distinct variations in maintenance hosts and the serovar carriage occur throughout the world. Knowledge about the prevalent serovars and their maintenance hosts is essential for understanding the epidemiology of the disease in any region.

#### **MODE OF TRANSMISSION TO THE HUMAN HOST:**

The transmission of leptospiral infection from animals to man occur by direct contact with blood, tissues and urine of infected animals or by indirect contact (more common) by exposure to an environment contaminated by leptospira (water & soil contaminated with infected urine).

Water-borne transmission has been documented; point contamination of water supplies has resulted in several outbreaks of leptospirosis. Inhalation of water or aerosols also may result in infection via the mucous membrane of the respiratory tract. Rarely infection may follow animal bites.

Direct transmission have been demonstrated .in human beings rarely However excretion of leptospire in human urine has been demonstrated many months after the recovery from the infection (Ballard et al, 1986). It is thought that the low pH of human urine limits the survival of leptospira after excretion.



Transmission by sexual intercourse during convalescence has been reported (Harrison et al, 1988)<sup>26</sup>.

There is also a significant risk associated with recreational exposures occurring in water sports (Mumford, C.J. 1989)<sup>42</sup> including swimming, canoeing, white water rafting and fresh water fishing.

Many sporadic cases of leptospirosis in tropical regions are acquired following avocational exposures that occur during the daily activities of life like barefooted walking in damp conditions, gardening with bare hands etc (Agarwal et al., 1986)<sup>1</sup>.

#### *Environment:*

Transmission of leptospiral infection depends to a great extent on the environment which favours the survival of leptospira outside the animal host. Optimal factors for the survival of leptospira are the presence of moisture, warmth (28-32<sup>0</sup>C), pH values of soil and surface water (pH 6.2 - 8). Under laboratory conditions, leptospires in water at room temperature remain viable for several months at pH 7.2 to 8 but in river water survival is shorter and is prolonged at lower temperatures. The presence of domestic sewage decreases the survival time to a matter of few hrs but in an oxidation ditch filled with cattle slurry, viable leptospires are detected for several weeks (Turner L.H, 1970)<sup>56</sup>.

#### **EPIDEMIOLOGICAL PATTERNS:**

Three epidemiological patterns of leptospirosis were defined by Faine (Faine S, 1994)<sup>19</sup>.

1. The first type occur in temperate climates where few serovars are involved and human infection almost always occur by direct contact with infected animals like cattle & pigs. Control by immunization of animals is potentially possible.
2. The second type occur in tropical wet areas within which there are many serovars infecting humans, animals and large number of reservoir species, including rodents, farm animals occupation but results more often from widespread environmental contamination particularly during the rainy season. Control of rodent population, drainage of wet areas & occupational hygiene are all necessary for prevention of human leptospirosis. These are the areas where large outbreaks are most likely to occur following floods, hurricanes or other disasters.
3. The third type comprises the rodent borne infection in the urban environment While this is of lesser significance in most parts of the world, it is potentially more important when the urban infrastructure is disrupted by natural disasters. This type of infection is now rarely seen in developed countries, but is exemplified by the recent rediscovery of urban leptospirosis (occurring in slum areas of developing countries).

#### **LABORATORY DIAGNOSIS OF LEPTOSPIROSIS:**

Leptospirosis is usually misdiagnosed as pyrexia of unknown origin (PUO), Viral fever (Hanta Virus) Malaria, Enteric fever, Influenza or Pyelonephritis, severe icteric illnesses such as Viral

hepatitis, Septicaemia with jaundice. Hence, it is important to confirm the clinical diagnosis of leptospirosis by suitable laboratory tests. It is also important that the laboratory diagnosis should always be correlated with the clinical signs and symptoms exhibited by the patients.

## **LABORATORY TESTS<sup>20</sup>**

### *Antigen Detection Tests:*

#### *Demonstration of Leptospire or its products:*

1. Microscopy
  - a. Dark field microscopy
  - b. Phase contrast microscopy
2. Staining
  - a. Silver staining
  - b. Immune fluorescence
  - c. Immuno peroxidase staining
3. DNA hybridisation Analysis
4. Polymerase chain reaction
5. Co-agglutination test

### *Antibody Detection Tests:*

#### *I. Genus specific tests:*

- Macroscopic slide agglutination test (MSAT)
- Indirect fluorescent antibody technique (IFAT)
- Indirect Hemagglutination test (IHA)
- Counter Immuno Electrophoresis (CIE)

- Complement Fixation Test (CFT)
- Enzyme Linked Immuno Sorbant Assay (ELISA)
- Micro Capsule Agglutination Test (MCAT)
- Lepto Dip Stick
- Lepto Lateral Flow Test
- Lepto DRI Dot

## ***II. Serogroup/ Serovar Specific Test:***

Microscopic Agglutination Test (MAT)

### ***Collection of Sample:***

Leptospire may be present in the blood and CSF in the first 7-10 days of illness and in the urine from the 10- 14 day of illness to 30 day of illness. Agglutinating antibodies appear in the blood towards the end of the first week, reaches the peak in the third or fourth week of illness and then gradually declines with low levels persisting for an indefinite period.

### **MICROSCOPIC DEMONSTRATION:**

Leptospire may be visualised in clinical material by dark-field microscopy or by immunofluorescence or light microscopy after appropriate staining. Dark field examination of body fluids such as blood, urine, CSF and dialysate fluid has been used but is both insensitive and lacking specificity. Approximately  $10^4$  leptospire/ml is necessary per low power field to be visible by dark-field microscopy. Microscopy of blood is of value only during the first few days of the acute illness when leptospiremia occurs. Dark field

microscopy of blood is also subject to misinterpretation of fibrin or protein threads, which may show Brownian movement (Faine S, 1999)<sup>20</sup>.

#### **STAINING METHODS:**

Staining methods have been applied to increase the sensitivity of direct microscopic examination. Silver impregnation method is usually performed which artificially thickens the organism with a deposit of silver. Fontana method for films and Levaditi's method for sections are classical methods of silver staining for leptospire (Coghlan J.D et al., 1997)<sup>10</sup>. These methods lack sensitivity & specificity as they stain other tissue structures like elastin & reticulin fibres as well.

The various other silver stains with slight modifications are Warthin - Starry technique, Young's modification and Van Orden method (Coghlan J.D. et al 1997)<sup>10</sup>. As with other visualisation techniques the presence of a large number of intact organisms is required. So staining methods are relatively insensitive and do not identify the serovar.

#### ***Culture methods:***

The infecting strain of leptospira can be isolated in culture if suitable specimen (blood or urine) is obtained before the administration of antibiotics (V. Sitprija, 1996)<sup>51</sup>. For routine purposes liquid or semisolid media are used. For cloning, purifying of contaminated cultures and research purposes solid media are used.

### *Liquid Media:*

It is used for growing leptospire intended for use as antigens in serological tests. It consists of a buffered salts base with or without peptone to which is added either rabbit serum as in Stuart or Korthoff's media or preferably serum derivatives (bovine serum) & polysorbate as in EM media (Ellinghausen & McCullough's media). A modification of EM media called EMJH (Ellinghausen, McCullough, Johnson & Harris) is available commercially (L.H. Turner, 1970)<sup>56</sup>.

### *Semisolid Media:*

It is prepared by adding 0.2 - .5% (w/v) agar to any suitable liquid media. Agar appears to favour the multiplication of leptopires & semisolid medium is used for isolating leptospira from blood, urine and from animal tissues. It needs less frequent subculturing when compared to liquid media. It helps to maintain the virulence of freshly isolated strains for a long time and it is also used for preparing cultures intended for intravenous inoculation into rabbits for production of hyperimmune serum<sup>20</sup>.

Isolation of leptospira from urine is affected by contamination with other microorganisms. Neomycin sulphate and 5-Fluorouracil are used to prevent the growth of contaminants. Leptospire multiply in the upper 2-3 cms of semisolid media in tubes with the development of maximum turbidity. A feature which appears in many cultures is the development of one or more distinct discs of turbidity known as Dinger's rings at varying depths in the column of the medium (Turner L.H. 1970)<sup>56</sup>.

Solid media like tryptose phosphate broth with agar is used for cloning and for other specialised purposes (Coglanet et al., 1997)<sup>10</sup>. Leptospire produce discrete hemispherical colonies just below the surface of medium in petri dish. The plates are to be kept for atleast 6 weeks before discarding them as negative as the colonies are slow to develop (Cox & Larsen, 1957)<sup>11</sup>.

Slow growth of organisms requiring prolonged incubation and increased risk of microbial contamination are the reasons which prevent cultural methods from being used as a routine laboratory method for the diagnosis of leptospirosis.

## **SEROLOGICAL METHODS**

Serological tests are the most commonly used test for routine diagnostic purposes. The following is the list of more commonly used tests.

1. Complement Fixation test (CFT)
2. Sensitised Erythrocyte lysis
3. Macroscopic slide agglutination ( MSAT)
4. Microscopic agglutination test (MAT)
5. Immunofluorescence test
6. Indirect Hemagglutination Test(IHA)
7. Counter Immuno electrophoresis
8. ELISA - IgM, IgG
9. Microcapsule Agglutination test
10. DOT- ELISA

11. IgM - Dipstick test
12. Latex Agglutination test
13. Lepto Dridot assay
14. Lateral flow assay

#### **MICROSCOPIC AGGLUTINATION TEST:**

The standard reference test for serological diagnosis of leptospirosis is the microscopic agglutination test. After incubation the serum antigen mixtures are examined microscopically for agglutination and the titres are determined. The MAT is a complex test to control, perform and interpret (Turner et al, 1970)<sup>56</sup>. Live cultures of all serovars required for use as antigen must be maintained. This applies to both the live formalin-killed antigens. MAT titers are affected by the culture medium in which the antigens are grown.

The range of antigens should include serovars representative of all serogroups and all locally common serovars. This test is read by dark-field microscopy. The end point is the highest dilution of serum at which 50% agglutination occurs. Because of the difficulty in detecting when 50% of leptospire are agglutinated, the end point is determined by the presence of approximately 50% free, unagglutinated leptospire compared to the control suspension. Interpretation of the MAT is complicated by cross reactions that occur between different serogroups samples collected during acute phase. The broad cross reactivity in the acute phase followed by relative serogroup specificity in convalescent phase samples results from the detection of both IgM-IgG antibodies by this test and the presence of several common antigens among leptospire<sup>20</sup>.



### **MACROSCOPIC SLIDE AGGLUTINATION TEST:**

This is a simple agglutination test commonly used in many laboratories for rapid diagnosis of leptospirosis at the genus level. It is useful in determining whether a patient is likely to be suffering from acute leptospirosis irrespective of the infecting strains. The antigens used for this test contain all the locally available pathogenic serovars along with *patoc* 1 strain antigen that have been killed by formalin or by heating in a boiling waterbath. The antigens used in the MSAT test react with a wider range of different antisera. This test is done by mixing a certain amount of concentrated killed antigen and the patients serum and they are allowed to react for a specified period. After the specified period the presence of agglutination is determined by the naked eye. This test is slightly less specific than MAT but it gives positive reaction earlier in the course of illness<sup>20</sup>.

### **OTHER SEROLOGICAL TESTS:**

The Lepto dipstick assay test detects the presence of specific IgM antibody which binds to broadly reactive antigen prepared from a non-pathogenic strain (Sehgal et al, 1995)<sup>49</sup>. The Lepto Dridot assay consists of coloured latex particles activated with broadly reactive leptospiral antibody that is dried onto an agglutination card. The assay is based on the binding of leptospira specific antibody present in the serum sample to the antigen causing a fine granular agglutination that tends to settle at the edge of the droplet (Smits et al., 2001)<sup>52</sup>.

ELISA (Enzyme linked immunosorbent assay) for the detection of IgM antibodies has been shown to be more sensitive than MAT when the sample was taken early in the acute phase of

the illness. IgM antibodies have been detected by ELISA in CSF from patients with icteric leptospirosis. ELISA methods have been applied with a number of modifications.

An IgM specific dot ELISA was developed in which polyvalent leptospiral antigen was dotted on to nitrocellulose filter discs in microfilter tray wells, allowing the use of smaller volumes of reagents. Further modifications of this approach have been used to detect IgG & IgA in addition to IgM tests. These tests have employed an immunodominant antigen and a polyester fabric resin support in place of nitrocellulose. A Commercial IgM-dot ELISA dipstick has been shown to be as sensitive as microtiter plate IgM-ELISA. (Gussenhoven et al, 1997)<sup>25</sup>.

An Indirect Hemagglutination assay (IHA) developed at CDC was shown to have sensitivity of 92% and specificity of 95% compared with the MAT. But later studies conducted at endemic areas showed lesser sensitivity.

A Microcapsule agglutination test using a synthetic polymer in place of red blood cells has been evaluated extensively in Japan & China. In an International multicenter evaluation, it was found to be more sensitive than the MAT and the IgM-ELISA in early acute phase samples, but failed to detect infections caused by some serovars. An advantage of this direct agglutination method is that it can be applied without modification to sera from animal species as well<sup>20</sup>.

Other methods like Immunofluorescence, Radio Immuno assay, Counter immunoelectrophoresis and Thin layer immunoassay are not widely used.

## **POLYMERASE CHAIN REACTION:**

PCR is an in-vitro technique that allows amplification of a specific deoxyribonucleic acid region that lies between the regions of known DNA sequences. PCR aids in the rapid diagnosis of leptospirosis as thus helping in initiating early treatment.

In 1989, G.J.J.M. Van Eys et al.,<sup>57</sup> applied and evaluated use of PCR for detecting leptospirosis in cattle urine. Primers for PCR were synthesised from clones derived from leptospira hardjo library. They found that boiling of samples or treatment with detergents were the effective methods of processing the sample. Urine samples containing fewer than 10 (Ten) leptospires gave positive results in PCR assay. This study reveals that an increase in annealing temperature allows fewer mismatches between primers and target DNA and will increase specificity of the test.

Merien et al in 1992<sup>41</sup> developed a PCR assay using four oligonucleotides. Primers in which a 331-bp sequence from the leptospira interrogans serovar canicola 16s gene was amplified and the PCR products were analysed by DNA-DNA hybridisation method. This test was able detect as few as 10 leptospires and the test was found to be suitable for detection of leptospires in clinical samples like blood, cerebrospinal fluid and urine.

P. Perolat et al (1993)<sup>35</sup> evaluated the use of PCR assay for detecting the presence of pathogenic leptospires in samples of aqueous humor from a patient with unilateral uveitis, by using a pair of primers designed from 16s RNA gene of leptospira species. The assay was able to detect the presence of the concentration of leptospires in the aqueous humor.

The authors reported that 'PCR' was a valuable sensitive assay in the diagnosis of belated ocular complications of leptospirosis where the diagnosis is presumptive and based on serological and epidemiological data.

Elite C. Romero et al (1998)<sup>14</sup> studied the use of PCR in CSF samples in patients with aseptic meningitis and reported that PCR was more sensitive than MAT and Igm-ELISA in early diagnosis of leptospirosis.

Woo et al (2002)<sup>60</sup> evaluated PCR using Taqman fluorogenic probes for leptospira detection. He concentrated on the use of Taqman probes for the differentiation and identification of leptospira species using pure isolates obtained from a reference culture collection.

Fernandes Nasin et al in 2003<sup>21</sup> evaluated nested PCR method for the diagnosis of leptospirosis. Primers were designed to amplify a 264 bp region within the Lip L32 gene. This technique showed to be very specific for pathogenic serovars because Lip L32 gene is absent from non-pathogenic leptospira or any bacteria. The primers used in this reaction amplified a 183bp region within the 264bp region. Sensitivity was greatly increased by this approach and the only drawback was the high risk of contamination by the amplican resulting in false positive results.

Raven E. Reitsletter et al, 2003<sup>45</sup> developed species specific PCR primer sets for the detection of leptospira in blood samples. For identification of individual leptospira species PCR primer that detect the OMPL 1 gene sequence of majority of pathogenic leptospires like *L.interrogans*, *L.kirschneri*, *L.weillii*, *L.nognchi*, *L.santarosai* and

*L.borgpetersemi* were developed. These primers did not cross react with each other and were of good epidemiological value.

Paul N. Levett et al 2005<sup>34</sup> developed a real time PCR assay using a 423 bp target on the Lip L32 gene. He used representative serovars from 16 (sixteen) species of leptospira. The analytical sensitivity of the assay was 3 genome copies per reaction in blood and approximately 10 (ten) genome copies per reaction in urine.

### **STAPHYLOCOCCAL CO-AGGLUTINATION TEST**

Co-agglutination is a serological event which permits the detection of minutes quantities of soluble antigen in clinical specimens and has been used to detect cell wall and capsular antigens on the bacterial pathogens or in fluids collected from the infected host

The Cowan-1 strain of *Staphylococcus aureus* (ATCC 12598) is very rich in protein A and can bind immunoglobulin of (IgG) non-specifically through the Fc (crystallisable part) of the immunoglobulin molecule. This leaves the Fab sites (antibody binding site) free to react with the homologous antigen. The reaction is visualised by the clumping or agglutinating reaction with *Staphylococci* on a glass slide, in a tube or directly on an agar plate. For example after streaking for isolation, colonies of bacteria could be identified directly on a petriplate by detecting this reaction after a suspension of *Staphylococci* conjugated with specific antibacterial IgG antibodies is added<sup>33</sup>. This procedure is very simple and does not require complex instrumentation or hazardous chemicals. It can be used as a routine screening technique in the clinical laboratory and can replace counter immunoelectrophoresis and slide agglutination tests.

Krook A & Holmberg H (1987)<sup>38</sup> used co-agglutination test for detecting *pneumococcal C-polysaccharide* in sputum samples and found it to be a rapid sensitive and specific test for the diagnosis of *streptococcal pneumoniae* in adults. The sensitivity of the test was 95.8% of specificity was 96.5%.

Rajalakshmi B & Kanungo R (2000)<sup>44</sup> reported staphylococcal co-agglutination method as a cost effective method of serotyping *Streptococcus pneumoniae*.

Robert J et al (1991)<sup>47</sup> reported that co-agglutination test was more sensitive than slide agglutination for the rapid identification of *Hemophilus influenzae* type b in various clinical samples

Firdausi Qadri et al (1994)<sup>22</sup> developed and evaluated rapid antibody based co-agglutination test for direct detection of *Vibrio cholerae* O 139 in 83 of 120 watery diarrhoeal stool samples. He reported 92% sensitivity and 100% specificity for this test conducted at International Centre for Diarrheal Research, Bangladesh.

Isia A. Mikhail et al (1983)<sup>28</sup> reported blood clot culture co-agglutination procedure for rapid, and economical diagnosis of enteric fever. He reported that co-agglutination test was effective as conventional blood culture methods with reported sensitivity of more than 95% in 95 samples tested .

Islam MN et al (1989)<sup>29</sup> developed and evaluated co-agglutination test to detect rota virus antigens in stool samples of children presenting with acute watery diarrhoeas. Rabbit antisera raised against RV5 & SA 11 *rotavirus* strains and coated with

*Staphylococcus* cowan 1 strain detected *rotavirus* antigens in 276 of the total 1332 samples tested. He reported that the sensitivity and specificity of the test was 76.19% & 89.66% respectively.

Mathur MS et al (1989)<sup>39</sup> compared co-agglutination test with Rotalex kit for latex agglutination for the detection of *rotavirus* antigen in stool samples of 80 cases and found that Co-Agglutination was positive in 20 cases as compared to 16 cases by latex agglutination test. He also reported 97.5% specificity for this test.

Khyriem AB et al (2003)<sup>33</sup> compared co-agglutination test with commercially available latex agglutination test microscopy and culture methods for *cryptococcal* meningitis and reported that co-agglutination test was found to be superior to latex agglutination test with proportionate positivity of 24% for co-agglutination and 14.7% for latex agglutination test. It can be used as a very useful adjunct to direct microscopy and culture for the diagnosis of *cryptococcal* meningitis (Indian Journal of Medical Microbiology

Ravinder PT, Parija SC and Subba Rao K.S.V.K.(2000)<sup>46</sup> reported Urinary antigen detection by co-agglutination method for rapid diagnosis of cystic *echinococcosis* in rural and field settings.

#### **TREATMENT:**

Penicillin, Streptomycin, Tetracycline Erythromycin and cephalosporins are among the antimicrobials capable of killing leptospires. Early institution of antimicrobial treatment may present complications like renal failure. Parenteral aqueous benzyl penicillin in a dosage of 1 mega units 6 hourly for a period of 1 week is recommended in adults. For patients with penicillin

hypersensitivity, tetracycline at a dosage of 2 g daily is recommended provided renal function is normal. Doxycycline (200 mg daily) and third generation cephalosporin like cefataxime or ceftriaxone given intravenously are also found to be highly effective<sup>26</sup>.

#### **PREVENTION:**

Prevention consists of avoiding or indirect exposure of humans to infected animal urine and tissues. Occupational hygiene, protective clothing and safe work practices are important in preventing the disease in working population.

Vaccines are effective in preventing the disease in animals. The outer membrane vaccine is more effective than the whole cell and protoplasmic cylinder vaccine. Its use on man is impracticable due to the presence of many serovars. However vaccination against a specific serovars prevalent in the area has been shown to be effective. Chemoprophylaxis with Doxycycline 200mg weekly prevents infection but is recommended only for short term use in high risk individuals<sup>20</sup>.



## **AIMS OF THE STUDY**

- To develop Co-agglutination test for the early diagnosis of leptospirosis.
- To evaluate Co-agglutination test as a diagnostic tool in the early diagnosis of leptospirosis as compared to Polymerase Chain Reaction (PCR).

# **MATERIALS AND METHODS**

## **STUDY PERIOD**

January 2006 - May 2007.

## **SAMPLE**

Blood samples from 100 patients with clinical features suggestive of Leptospirosis (Fever, Headache, Muscle pain, Conjunctival suffusion) were included in the study. Blood samples were collected during the early period of illness (3-5 days) and were subjected to DFM, PCR, Co-agglutination test MSAT & MAT. Second samples were collected 4-6 days after the first sample and were subjected to MSAT & MAT tests.

## **SOURCE OF SAMPLES**

The samples were received from patients attending Leptospirosis Research Cell (LRC), Institute of Microbiology, Madras Medical College, Chennai - 3.

## **METHODS**

Darkfield microscopic examination (DFM) was done using plasma for detecting live leptospire and Polymerase Chain Reaction (PCR), Co-Agglutination Test, Microscopic Agglutination Test (MAT), Macroscopic Slide Agglutination Test (MSAT) were done using serum samples collected from patients.

PCR was done using PCR diagnosis kit for *Leptospira* obtained commercially (Genei, Bangalore) for detecting leptospira DNA. The primers G1 & G2 were supplied with the same kit. PCR

tests were done at Dept. of Microbiology, Bharathidasan University, Trichy.

Rabbit antisera raised against leptospira were obtained commercially from Animal House, Dr. M.G.R. - Janaki College of Arts & Science for Women College Chennai - 28. for performing the Co-agglutination test.

*Leptospiral serovars autumnalis. australis ilcterohaemorrhagiae,. louisiana, gripphotyphosa, hebdomadis* & non - pathogenic serovars. *patoc* were used for MSAT & MAT. The above standard research strains were obtained from KIT & Royal Tropical. Inst, Netherlands and subcultured periodically in LRC, Institute of Microbiology, MMC, Chennai - 3.

## **MEDIUM**

EMJH medium was used for subculturing the organism for maintenance and antigen preparation. Ellinghausen -Mc cullough - Johnson Harris (EMJH) media is supplied as base and as enrichment.

### **EMJH BASE - (DIFCO LABORATORIES, USA)**

Ingredients	gms/lit
Sodium phosphate diphasic	1
Potassium	3
Sodium chloride	1
Ammonium chloride	.25
Thiamine	.005

**EMJH ENRICHMENT:**

The enrichment bovine serum albumin (BSA) at the proportion of 1gm of BSA per 10 ml of EMJH medium at a final concentration of 10%. It was then, filtered through a membrane (Pore size .22 $\mu$ ) and was added as enrichment after cooling.

**EMJH LIQUID MEDIUM (JOHNSON 1984)**

Liquid EMJH medium was prepared by dissolving 2.3gms of EMJH base in 900ml of triple distilled water, the pH is adjusted to 7.5. After autoclaving at 15 lbs for 15mts the medium was added aseptically with enrichment at 10% level. 100ml of enrichment is added to 900ml of EMJH base medium. Later it was distributed aseptically in screw capped tubes. It was checked for sterility after 24 hrs at 27<sup>0</sup>C.

**EMJH SEMISOLID MEDIUM:**

To prepare EMJH semisolid medium 0.2% bacteriological agar was added to the EMJH base and the complete medium was prepared as above.

**SELECTIVE AGENT:**

5-Fluoro uracil was used as selective agent. Leptospira are resistant to the antibacterial activity of pyrimidine analogue, 5-Fluorouracil  
(S. Faine, 1982).

100ml of 5FU was added to 5ml of distilled water. To this 0.1 to 0.2ml of 0.1N NaOH was added and heated gently to dissolve 5-FU completely. The pH was adjusted to 7.4 - 7.6 and the volume

was made upto 10ml with distilled water. The solution was sterilised by filtration through membrane filter (Pore size 0.45  $\mu\text{m}$ ).

Add 1ml of this into 100ml of EMJH medium to get the final concentration of 100 $\mu\text{gm}$  per ml.

#### **CULTURING OF LEPTOSPRIA:**

Appropriate amount of the organism was transferred to EMJH liquid and semisolid media. A period of 7 days incubation at room temperature (25-31 $^{\circ}\text{C}$ ) was maintained. The culture was then examined under DFM to check for the required growth and active motility of leptospira. Growth was also visualised as one or more rings, several millimetre below the surface of the semisolid medium. Once growth was found optimal it was used as live antigen.

#### **IDENTIFICATION OF LEPTOSPIROSIS:**

Plasma & serum samples were subjected to the following tests.

- DFM
- PCR
- Co-agglutination test
- MSAT
- MAT

The results from the above tests were compared to identify the most sensitive and early diagnostic method for leptospirosis.

### **DARK FIELD MICROSCOPY EXAMN (FAINE, 1982)<sup>18</sup> (DFM).**

5ml of blood was collected, treated with an anticoagulant (1% sodium oxalate) and centrifuged at 1000rpm for 15 mts. 10ml of plasma was placed on a thin microscopic slide and a coverslip was placed over it. Then the slide was examined under darkfield microscope with low and high power magnification.

### **POLYMERASE CHAIN REACTION:**

PCR involves the enzymatic amplification of DNA in vitro and was originally developed by Cetus corporation. This method is capable of increasing the amount of the target DNA sequence in a sample by synthesising many copies of DNA segment. PCR is carried out in discrete cycles and each cycle of amplification can double the amount of target DNA. The target DNA is exponentially amplified so that after 'n' cycles there are  $2^n$  times as much target DNA as was present initially.

#### *Components of PCR:*

##### *Oligonucleotides:*

Oligonucleotides used in this procedure were only 20 nucleotides in length because they were too short to form stable hybrids at the temperature used for polymerisation. Oligonucleotides were used at a concentration of  $1\mu\text{mol}$  was sufficient for 30 cycles of amplification.

Oligonucleotides used in this protocol were

G<sub>1</sub>.5' CTG AAT CGC TGT ATA AAA GT-3'

G<sub>2</sub>.5' GGA AAA CAA ATG GTC GGA AG-3'

*Buffers used:*

Composition of standard buffer (pH 8.3)

KCl	50mM
Tris HCl	10mM
MgCl <sub>2</sub>	1.5mM

The presence of divalent cations is critical because the optimal concentration of Mg<sup>2+</sup> is low. DNAs used as templates were dissolved in low Ionic Tris EDTA (LTE) Buffer.

LTE Buffer contains

- Tris HCl 10 mM (pH 7)
- EDTA (pH 8) 6.1 mM

The concentration of Mg<sup>2+</sup> optimised when ever a new combination of target and primer was used and also when deoxynucleotide triphosphate (d NTP's) primers were altered.

*Taq DNA Polymerase:*

Two forms of Taq polymerases were used. A native enzyme purified from *Thermus aquaticus* and a genetically engineered form of enzyme synthesized in *E.coli* (Amplitaq) Both forms of the polymerases carry a 3' to 5' exonuclease activity. Approximately 2 units of either of these enzymes were required to catalyse a typical PCR

*Deoxynuccetide Triphosphate (DNTP'S)*

Deoxy nucleotide Triphosphate (dNTP's) was used at saturating concentration (200ml of each dNTP's) A stock solution of d NTP's (50mm) were adjusted to pH7 with 1N NaOH to ensures

that the pH of the final reaction dose not fall below 7% PCR assay was carried out as per the method of Grave Kemp.

#### **SAMPLE PREPARATION**

- 1) 2.5ml of sample and 7.5ml of PBS was centrifuged. at 10,000 rpm for 25mts .
- 2) Pellet was resuspended in 5ml of distilled water and washed twice and the final pellet was re-suspended in 20 $\mu$ l of distilled water
- 3) Serum samples were heated to 96°C for 10 mts for denaturation and kept on ice (snap cooled) .
- 4) 4.5 $\mu$ l of this sample was taken for PCR assay.

#### **PCR PROCEDURE**

- Amplification of DNA was performed in a total volume of 50 $\mu$ l reaction mixture.
- The reaction mixture contains 5ml of 10x buffer (50mM KCl, 20mM MgCl<sub>2</sub> 10mM Tris HCl pH 8.3)
- The freeze dried primers G<sub>1</sub>,G<sub>2</sub> were mixed with nuclease free water to prepare stock solutions. From this primer stock solutions 100mM each were added to the reaction mixture.
- 100 mM each of the four deoxynucleotide Triphosphate (ATP, GTP, CTP, TTP totally 400 mM) were added.



- To this reactions mixture 3µl Taq DNA polymerase and 5µl of sample were added and required amount of distilled water (31ml) was added to make up the volume to 50ml.
- Amplification was carried out in MJ research DNA thermal cycler for 34 cycles with initial denaturation of DNA for 6.5mts at 94°C, then subsequently. for 2mts at 94°C, for each cycle. Annealing of the primer was done for 1minute at 55°C and elongation of chain for 2mts at 72°C. After the completion of necessary cycles, a warming up period of 7mts was given.
- The PCR products were analysed by gel electrophoresis at pH 8.3 (Van Eyes et al, 1988)<sup>57</sup>.
- After the electrophoresis was completed the gel was viewed in UV-Reader. PCR products and molecular weight markers are seen as bright fluorescent adducts due to binding adduction of Ethidium bromide to DNA.

#### **INTERPRETATION OF THE RESULT:-**

In positive amplification, a single PCR product band at 285 base pair was obtained.

#### **CO-AGGLUTINATION TEST FOR LEPTOSPIROSIS (ANTIGEN DETECTION)**

**(Parija SC et al 2005 IV National workshop on simple diagnostic methods in Infectious diseases, JIPMER).**

Staphylococcal Co-agglutination is a procedure for the diagnosis & demonstration of leptospiral antigen in the blood during

the early phase of the disease. This test is based on the principle that staphylococcus aureus cowan I strain (SAPA) contains protein A which has an affinity for the Fc portion (Crystallisable part) of the Ig-G antibody. SAPA cells coated with leptospiral antisera will detect the antigen present in infected patients blood.

#### *Stages of the test*

- 1) Preparation of Antiserum
- 2) Preparation of SAPA cells.
- 3) Sensitisation of SAPA cells.
- 4) Performing the Co-Agglutination test.

#### *I. Preparation of Antisera:-*

Rabbit antisera raised against leptospiral antigen were obtained commercially from the Animal house of Dr. M.G.R. Janaki College of Arts & Sciences, R.A.Puram, Chennai-600 028. during the study period.

#### *Selection of Animals:*

Two young healthy male rabbits (Newzealand Strain) weighing approximately 3-4 kg were used. The rabbits were pretested for leptospiral antigen and antibodies by Dark-field microscopy and Macroscopic slide agglutination test respectively.

#### *Antigen used:*

Well grown live cultures of leptospira containing  $2 \times 10^8$  organism/ml (5-7day old) were used as antigens. The serovars

used includes the following: *icterohaemorrhagiae*, *autumnalis*, *australis*, *patoc hebdomadis*, *grippotyphosa*, & *louisiana*.

***Animal inoculation:***

The following is a schedule recommended by Kmety for the production of antisera to heat labile as well as the heat stable antigens (Faine et al., 1982).

Rabbits were injected intravenously in the marginal vein of ear according to the following schedule.

<b><i>Day</i></b>	<b><i>0</i></b>	<b><i>6</i></b>	<b><i>11</i></b>	<b><i>16</i></b>	<b><i>21</i></b>
Dose of live leptospiral culture injected in ml	1	2	4	6	6

At the end of 1 week after giving the last dose 10ml blood was collected from ear veins of each rabbit and serum separated. Antisera was purified by sodium sulphate precipitation method. The antisera was stored in small aliquots at  $-20^{\circ}\text{C}$ .

**2. PREPARATION OF SAPA CELLS:**

It includes the following three steps:

- Cultivation & Harvesting of *Staphylococcus aureus* Cowan I strain
- Sensitisation of SAPA cells
- Storage

**CULTIVATION & HARVESTING OF THE SAPA CELLS:**

***Requirements:***

- *Staphylococcus aureus* (Cowan I strain)

- Mueller Hinton agar slopes
- PBS ( pH 7.2)
- PBS containing .05% sodium azide,
- PBS containing .5% sodium azide
- 1.5% Formaldehyde
- 0.05% sodium azide
- Centrifuge tubes
- Water bath set at 80<sup>0</sup>C.

SAPA cells were grown on Mueller -Hinton agar slopes aerobically.

Harvesting of the cells was done as follows:

1. To the slope 3-5 ml PBS was added & shaken well.
2. It was centrifuged at 5000 rpm for 5 mts & supernatant discarded.
3. The pellet was washed thrice with PBS (PBS with 0.5% Sodium azide)
4. It was then fixed with 10 volumes of 1.5% formaldehyde in PBS (pH 7.2) at room temperature for 90 mts.
5. It was then washed thrice with PBS (7.2) and in 10 volumes of PBS containing sodium azide (0.05%).

6. It was then heated at 80<sup>0</sup>C for 5 mts in a water bath.
7. It was then washed thrice with PBS (7.2). 10% suspension of SAPA cells with PBS with Sodium Azide 0.05% was then prepared.
8. It was then divided and stored in alequots at -20<sup>0</sup>C.

#### **SENSITIZATION OF CELLS:**

##### *Requirements:*

- Hyperimmune sera
- SAPA cells
- Water bath set at 30<sup>0</sup>C.
- PBS containing 0.1% Sodium Azide
- PBS (pH 7.2)

##### *Procedure:*

Sensitisation with SAPA cells with hyper immune serum was done immediately after the preparation of SAPA cells. The steps involved are as follows:

1. 0.5ml of antiserum was added to 0.5 ml of 10% suspension of the SAPA cells.
2. It was mixed thoroughly and incubated at 37<sup>0</sup>C for 30 mts.
3. It was then washed once with PBS (pH 7.2) and re-suspended to a concentration of 2% with PBS (7.2) containing 0.1% Sodium Azide.
4. 2% suspension of sensitised cells was used as control.

## **PERFORMING THE CO-AGGLUTINATION TEST:**

### ***Requirements:***

- Sensitised SAPA reagent
- Unsensitised SAPA reagent
- Positive control
- Negative Control
- Glass slide
- Micropipette
- Discarding jar.

### ***Procedure:***

1. A clean glass slide was used for performing the test.
2. The slide was divided into two halves
3. A drop of the test serum was added to each half
4. The same volume of 2% sensitised SAPA cells was added to one half and unsensitised SAPA cells was added to the other half (control)
5. The slide was rotated manually for 2 mts and then inspected.
6. Appropriate controls were examined in parallel with each test.

### *Interpretation:*

Agglutination with sensitised SAPA cell suspension and not with unsensitised SAPA cell suspension is taken as a positive result.

Positive: Indicates the presence of Antigen.

Negative: Indicates the absence of antigen.

### **MACROSCOPIC SLIDE AGGLUTINATION TEST (MSAT) (MAZZONELDI ET AL., 1974)<sup>40</sup>**

1. Different serovars of leptospira were grown in EMJH liquid medium for 7 days at 30<sup>0</sup>C in a shaking incubator.
2. After checking for growth & purity the leptospire were killed with formalin. (0.5ml of formaldehyde in 100ml culture).
3. After 30mts the killed leptospira culture was kept in boiling water bath for 30mts.
4. The culture was rotated at every 15 mts.
5. After cooling to room temperature, the culture was centrifuged for 30 mts at 10,000 rpm.
6. The supernatant was used as antigen. A pooled suspension of locally prevalent serovars in Chennai namely Icterohaemorrhagiae, Autumnalis, Australis, Louisiana, Grippotyphosa, Hebdomadis & non-pathogenic Patoc were used as antigens in MSAT.

*Preparation of Phosphate buffer saline (PBS) (pH 7.2)*

NaCl	8gm/liter
K <sub>2</sub> HPO <sub>4</sub>	1.12gm/liter
KH <sub>2</sub> PO <sub>4</sub>	0.34gm/liter
Distilled water	1liter

*Procedure:-*

- 8µl of PBS was added to all the depressions of the slide.
- Then 11µl of the prepared antigen was added to all the depressions of the slide.
- Negative serum added with antigen and PBS was used as negative control.
- Positive serum added with antigen and PBS. It was used as positive control.
- The last depression containing saline & antigen was used as antigen control.
- 4ml of patients serum was added to the depressions in the slide
- The slide was placed in a rotator (180rpm) for 8mts.
- The slide was viewed macroscopically for the presence of any agglutination clumps and was confirmed in DFM.
- The results of MSAT were read as follows:



1. Clumps of Agglutination with complete clearing of leptospiral antigen	4+
2. Obvious Agglutination with partial clearing of antigen suspension	3+
3. 50% Agglutination	2+
4. 25% Agglutination	1+

No agglutination & uniformity of serum antigen mixture - Negative.

***Interpretation:***

An agglutination of  $\geq 2$  is considered as positive.

**MICROSCOPIC AGGLUTINATION TEST (SULZER & JONES, 1973)<sup>54</sup>**

The MAT was performed with seven (7) live cultures using *icterohaemorrhagiae*, *australis*, *autumnalis*, *hebdomadis*, *grippotyphosa*, *louisiana* & non-pathogenic *patoc* using standard microtitre methodology. The sera were initially screened at dilution of 1:20 and those that were positive was titrated further upto the end point. The highest dilution of serum that agglutinated 50% of leptospires under dark field microscopy were presumed to represent the titre of antibody specific for the particular serogroup used. When two or more serogroups react at the same (highest) titre, the result was recorded as mixed equal. Controls were put up for each one of the battery of the antigens used in the test.

***Procedure:***

The sera diluted to 1:10 in separate test tubes.

- 25 $\mu$ l of PBS was added to all the wells in the microtitre plate.

- The sera was diluted by doubling dilution as 1:20, 1:40 upto 1:320.
- Last well served as a control containing only PBS and antigen.
- 25µl of antigen was added to each well in the row (Each row of the microtitre plate was used for each serovar from the panel).
- The serum antigen mixture then mixed by gentle agitation and it was kept at room temperature for 2 hrs.
- One drop from each well was examined from lower dilution onwards after examinig the antigen control.
- By dark field microscopy, agglutination masses & free organisms were looked for.

***Interpretation of results:***

The highest dilution of serum antigen mixture, which showed 50% agglutination was taken as the end point titre of the serum for that particular antigen.

***Criteria for diagnosis:***

An initial titre >1:80 or a four fold rise in titre of MAT was considered as positive.

## RESULTS

*Total no of samples screened : 100*

- DFM, PCR, Co-agglutination, MSAT & MAT test were done during early febrile period (3-5days)
- Secondary screening was done during follow up in the late febrile period (9-12 days) by MSAT & MAT

The results of these test are as follows:

**TABLE - I :**  
**PRESENTING CLINICAL FEATURES (N=36)**

<b>S.No</b>	<b>Clinical feature</b>	<b>% of patients</b>
1.	Fever	100%
2.	Myalgia	55%
3.	Headache	40%
4.	Conjunctival suffusion	15%
5.	Icterus	5%
6.	Uremia	2%
7.	Others	10%

Fever was the predominant symptom followed by Myalgia & Headache.

**TABLE - II:**  
**AGE DISTRIBUTION IN POSITIVE CASES OF LEPTOSPIROSIS**

<b>S.No.</b>	<b>Age group (in yrs)</b>	<b>No. of positive cases</b>	<b>% Percentage</b>
1.	0-20	9	26
2.	21-40	15	42
3.	>40	12	32

Most cases were from 21-40 age group.

**TABLE – III:  
SEX DISTRIBUTION IN POSITIVE CASES OF LEPTOSPIROSIS**

<b>S. No</b>	<b>Sex</b>	<b>Male</b>		<b>Female</b>	
		<b>No. of Positive Cases</b>	<b>%</b>	<b>No. of Positive Cases</b>	<b>%</b>
1.	Total no of samples screened	21	58	15	42

**TABLE-IV:  
RESULTS OF VARIOUS DIAGNOSTIC TESTS  
IN EARLY FEBRILE PERIOD (3-5 DAYS) (N=100)**

<b>Test</b>	<b>No of. Positive cases</b>
PCR	36
CO-agglutination	30
DFM	10
MSAT	12
MAT	8

PCR was the most sensitive test (positive in 36 case) followed by Co-agglutination test. (30 cases)

**TABLE – V:  
COMPARISON OF RESULTS OF PCR,  
CO-AGGLUTINATION & DFM DURING EARLY FEBRILE PERIOD  
(3-5 DAYS) (N=100)**

<b>Test</b>	<b>No of. Positive Case</b>
PCR	36
CO-AGGLUTINATION	30
DFM	10

**TABLE–VI :**  
**COMPARISON OF CO- AGGLUTINATION TEST WITH**  
**MSAT & MAT DURING EARLY FEBRILE PERIOD**  
**(3-5 DAYS) (N=100)**

<i>Test</i>	<i>Total no of. positive case</i>
CO-AGGLUTINATION	30
MSAT	12
MAT	8

Co-agglutination test had better sensitivity than MSAT& MAT.

**TABLE-VII:**  
**MSAT RESULTS DURING EARLY (3-5DAYS) &**  
**LATE (9-12 DAYS) FEBRILE PERIOD (N=100)**

<i>Febrile period in days</i>	<i>Degree of positive agglutination</i>			<i>Total</i>	<i>Percentage</i>
	<i>2+</i>	<i>3+</i>	<i>4+</i>		
3-5 DAYS	8	4	–	12	33%
9-12 DAYS	20	8	6	34	94%

MSAT was more sensitive during the late febrile period.

**TABLE - VIII:**  
**MAT RESULTS DURING EARLY (3-5 DAYS)&**  
**LATE FEBRILE PERIOD (9-12 DAYS) (N=100)**

<i>Febrile period in days</i>	<i>Positive Titre</i>			<i>Total no of. Positive case</i>	<i>Percentage</i>
	<i>1/80</i>	<i>1/160</i>	<i>1/320</i>		
3-5 DAYS	6	2	–	8	22 %
9-12 DAYS	14	10	8	32	88 %

MAT was more efficient test during the late febrile period.

**TABLE -IX:  
MAT POSITIVE TITRE IN VARIOUS SEROVARS  
(EARLY FEBRILE PERIOD) (3-5 DAYS)**

<b>Serovar</b>	<b>Positive Titre</b>			<b>Total</b>
	<b>1/80</b>	<b>1/160</b>	<b>1/320</b>	
L. ICTEROHAEMORRHAGIAE	3	-	--	3
L. GRIPPO TYPHOSA	--	--	--	--
L. HEBDOMADIS	--	--	--	--
L. AUSTRALIS	2	--	--	2
L. AUTUMNALIS	1	--	--	--
L. PATOC	2	--	--	3
L. LOUISIANA	--	--	--	--
			<b>Total</b>	<b>8</b>

Serovars *icterohaemorrhagiae* & *patoc* were the predominant serovars isolated.

**TABLE-X:  
MAT POSITIVE TITRE IN VARIOUS SEROVARS  
(LATE FEBRILE PERIOD) (9-12 DAYS)**

<b>Serovar</b>	<b>Positive titre</b>			<b>Total</b>
	<b>1/80</b>	<b>1/160</b>	<b>1/320</b>	
L. ICTEROHAEMORRHAGIAE	3	5	3	11
L. GRIPPO TYPHOSA	1	1	0	2
L. HEBDOMADIS	-	-	--	-
L. AUSTRALIS	2	4	2	8
L. AUTUMNALIS	--	1	-	1
L. PATOC	2	6	1	9
L. LOUISIANA	-	1	-	1
<b>TOTAL</b>	<b>8</b>	<b>18</b>	<b>6</b>	<b>32</b>

*Icterohaemorrhagiae* (11), *patoc* (9) and *australis* (8) were the most common serovars..

Table-1 shows the presenting clinical features. Fever was the major clinical feature (95%) followed by myalgia (55%) headache (45%) and conjunctival suffusion (15%) other symptoms included GIT symptoms and Atypical pneumonia etc.

Table-2, It is interesting that infection was more common in working age group (21 - 40 Years) than in the younger age group (<21 Years)

Table-3 showed that 58% of positive cases were males and 42 % were females.

Table 4 the comparative analysis of the various diagnostic tests in the early febrile period. PCR showed the highest positivity of 36 cases out of 100 followed by Co-agglutination test (30) MSAT (12) DFM 10 and MAT(8).

Comparative analysis of PCR, Co-agglutination & DFM during the early febrile period is shown in Table–5. Sensitivity of Co-agglutination & DFM when compared to PCR were 85.7% and 27.5% respectively. (Table-5).

Co-agglutination test showed higher sensitivity of (85.7%) when compared to MSAT (33% ) and MAT (22%) in the early febrile period. (Table 6)

Table 6 Secondary screening of samples by MSAT and MAT during the late - febrile period (9-12 days) showed that MSAT was slightly more sensitive (94%) than MAT (88%).

Table 7: MSAT was positive in 33% cases early febrile period (3-5 days) and 94% positive in repeat samples taken during the late febrile period (9-12 days) Degree of agglutination was 2+ for most of the cases.

Microscopic agglutination test was positive in 22% of cases during early febrile period and 88% during the late febrile period (Tab 8).

Table 9 Shows serovar specific distribution of samples in MAT positive cases and also their titre of positivity. During the early febrile period 8 cases were positive. *Icterohaemorrhagiae* & *patoc* were the predominant serovars followed by *australis*.

During the late febrile period 32 cases were positive and out of which 8 cases showed positive agglutination in 1/80 dilution of serum, 18 cases in 1/160 dilution & 6 cases in 1/320 dilution, *Icterohaemorrhagiae* was the predominant serovar isolated followed by *patoc* (9) & *australis* (8) (Table 10).

#### **EVALUATION OF CO-AGGLUTINATION AS A DIAGNOSTIC TEST CRITERIA FOR EVALUATION:**

##### ***Sensitivity:-***

It is the probability that the test result will be positive when the disease is present. (true positive rate expressed as a percentage.)

$$\begin{aligned}\therefore \text{Sensitivity} &= \frac{\text{True positive}}{\text{True positive} + \text{false negative}} \times 100 \\ &= \frac{36}{36+6} \times 100 = 85.7\%\end{aligned}$$



### *Specificity:-*

It is the probability that a result will be negative when the disease is not present (true negative rate expressed as a percentage)

$$\begin{aligned}\text{Sensitivity} &= \frac{\text{True Negative}}{\text{True negative} + \text{false positive}} \times 100 \\ &= \frac{64}{64+4} \times 100 = 94.11\%\end{aligned}$$

### *Positive Predictive Value (PPV):*

It is the probability that a person is infected when a positive result is obtained. In practice predictive valves should only be calculated from studies that legitimately reflect the number of people in that population who are infected with the disease of interest at time.

This is because predictive values are inherently dependent upon the prevalence of infection. Thus positive predictive value is calculated using the formula.

$$\begin{aligned}\text{PPV} &= \frac{\text{True positive}}{\text{True positive} + \text{false positive}} \times 100 \\ &= \frac{36}{36+4} \times 100 = 90\%\end{aligned}$$

### *Negative predictive value (NPV):-*

The negative predictive value of a test is the probability that a person is not infected when a negative test result is obtained.

$$\text{NPV} = \frac{\text{True negative}}{\text{True negative} + \text{false negative}} \times 100$$

$$= \frac{64}{64+6} \times 100 = 91.4\%$$

***Positive Likelihood Ratio (PLR):-***

It is the ratio between the probability of a positive test result, given the presence of the disease and the probability of a positive result, given the absence of the disease.

$$\begin{aligned} \text{PLR} &= \frac{\text{True positive}}{\text{false positive}} \\ &= \frac{36}{4} = 9 \end{aligned}$$

***Negative likelihood Ratio (NLR):-***

It is the ratio between the probability of a negative test result given the presence of the disease and the probability of a negative test result given the absence of the disease.

$$\begin{aligned} \text{NLR} &= \frac{\text{False Negative}}{\text{True Negative}} \\ \text{NLR} &= \frac{6}{64} \\ &= 0.93 \end{aligned}$$

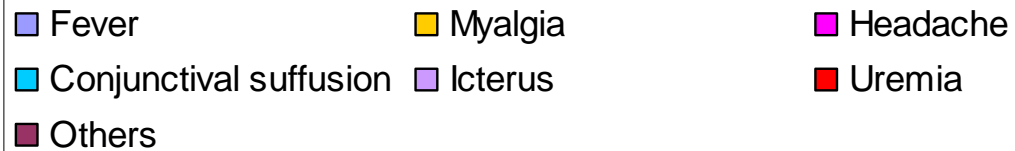
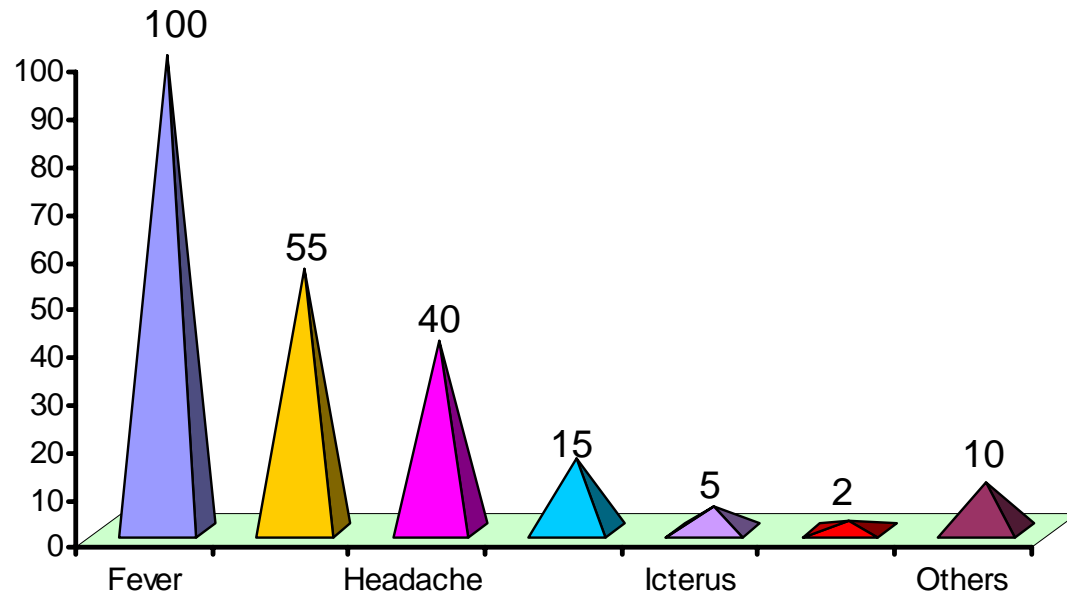
*Various criteria used to evaluate staphylococcal co-agglutination as a diagnostic test for leptospirosis:*

<b>S.No</b>	<b>Criteria for evaluation</b>	<b>Values obtained</b>
1.	Sensitivity	85.7%
2.	Specificity	94.1%
3.	Positive predictive value	90%
4.	Negative predictive value	91%
5.	Positive likelihood ratio	9
6.	Negative likelihood ratio	0.93

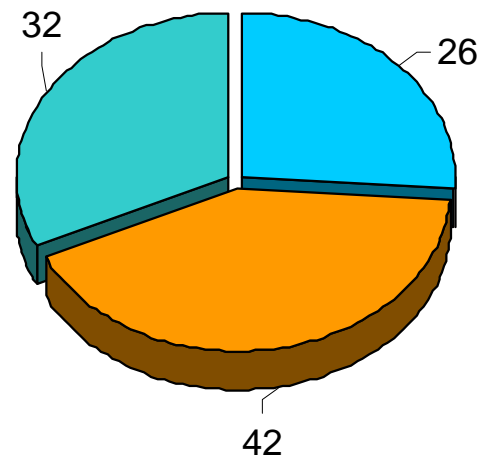
Mostly useful test will have negative likelihood ratio less than 1 (One) & less useful test will have a higher NLR value.

Since the value obtained in this study is less than 1 (One) & the sensitivity & specificity are also on the higher side it is considered that Staphylococcal co-agglutination test can be used as a diagnostic test for the detection of leptospirosis .

## PRESENTING CLINICAL FEATURES

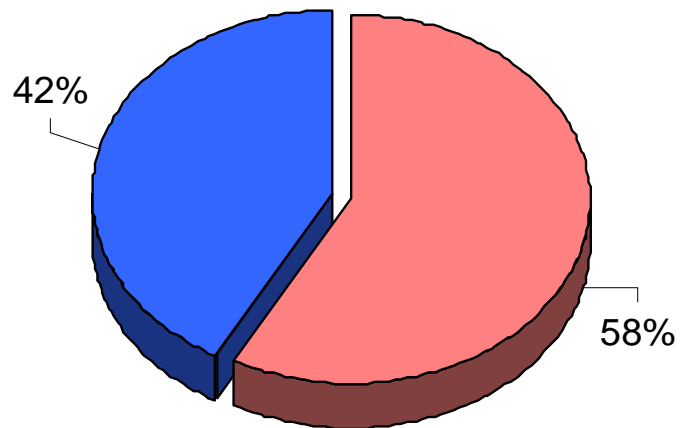


## AGE DISTRIBUTION



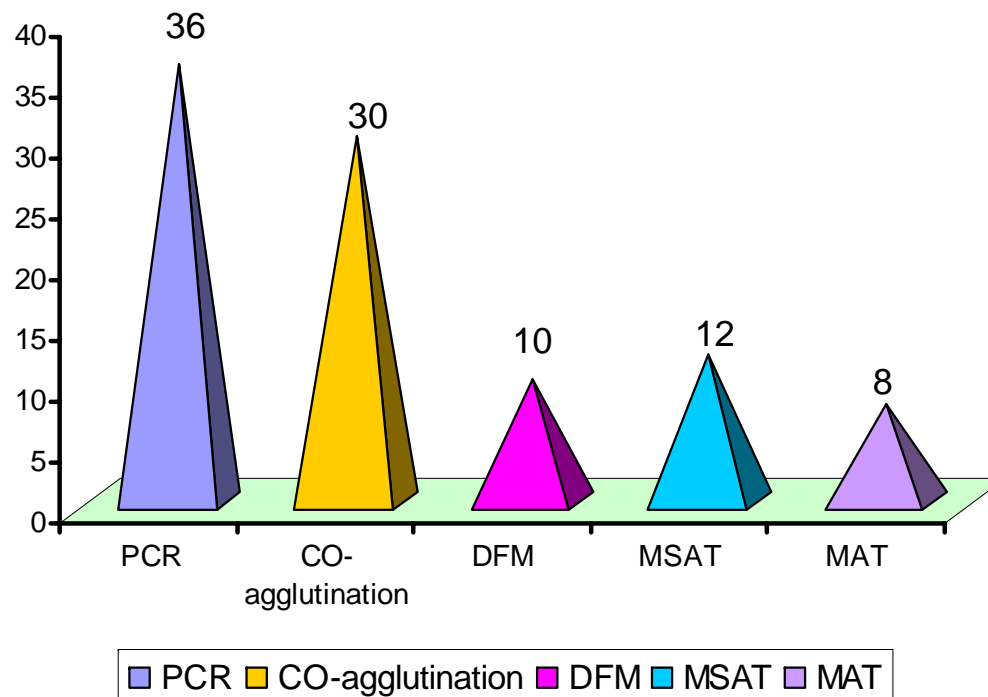
■ 0-20 Years ■ 21-40 Years ■ >40 Years

## SEX DISTRIBUTION

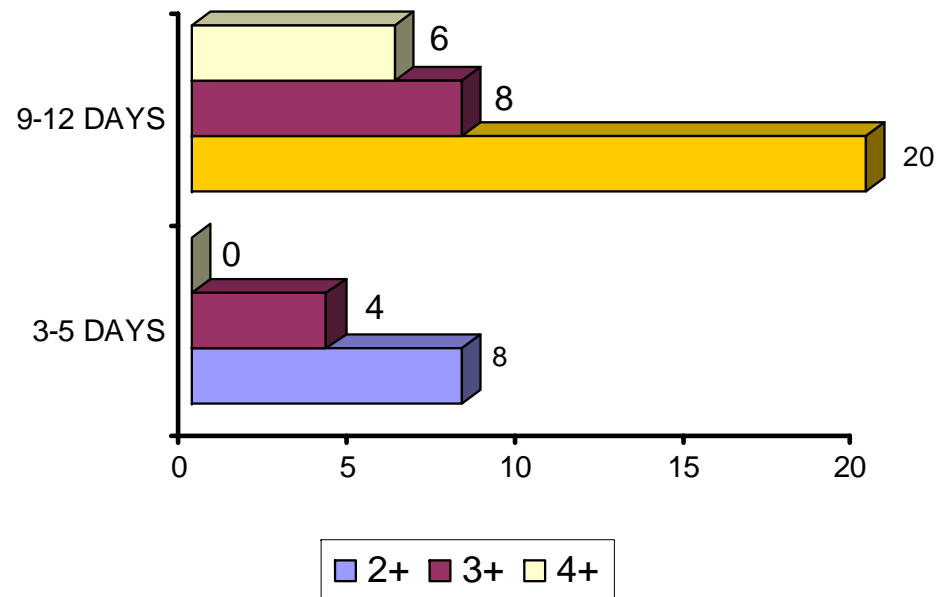


Male Female

## RESULTS OF VARIOUS DIAGNOSTIC TESTS IN THE EARLY FEBRILE PERIOD (3-5 DAYS)

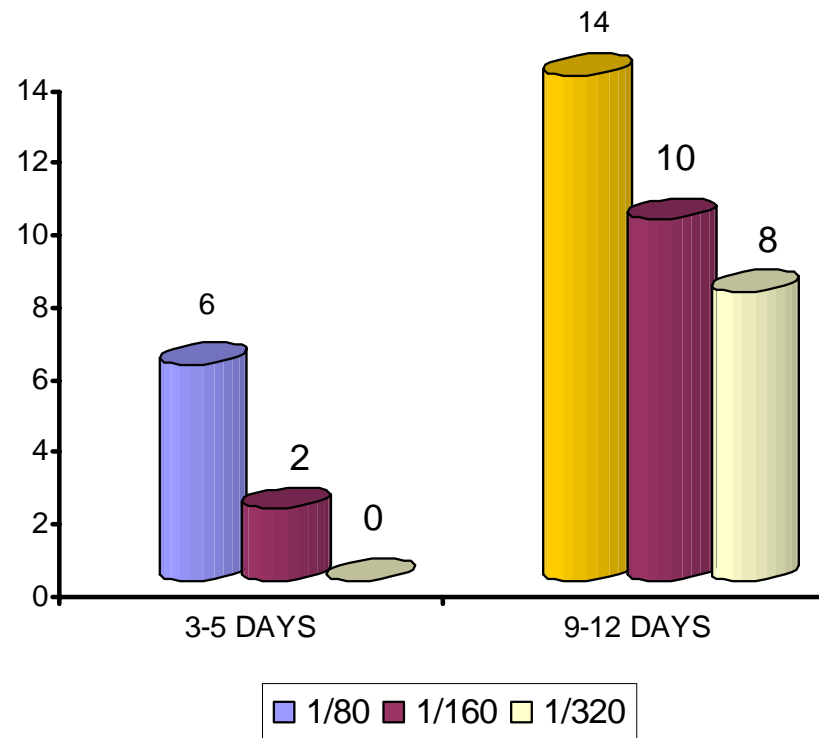


## MSAT RESULTS DURING EARLY (3-5 DAYS) & LATE (9-12 DAYS) FEBRILE PERIOD

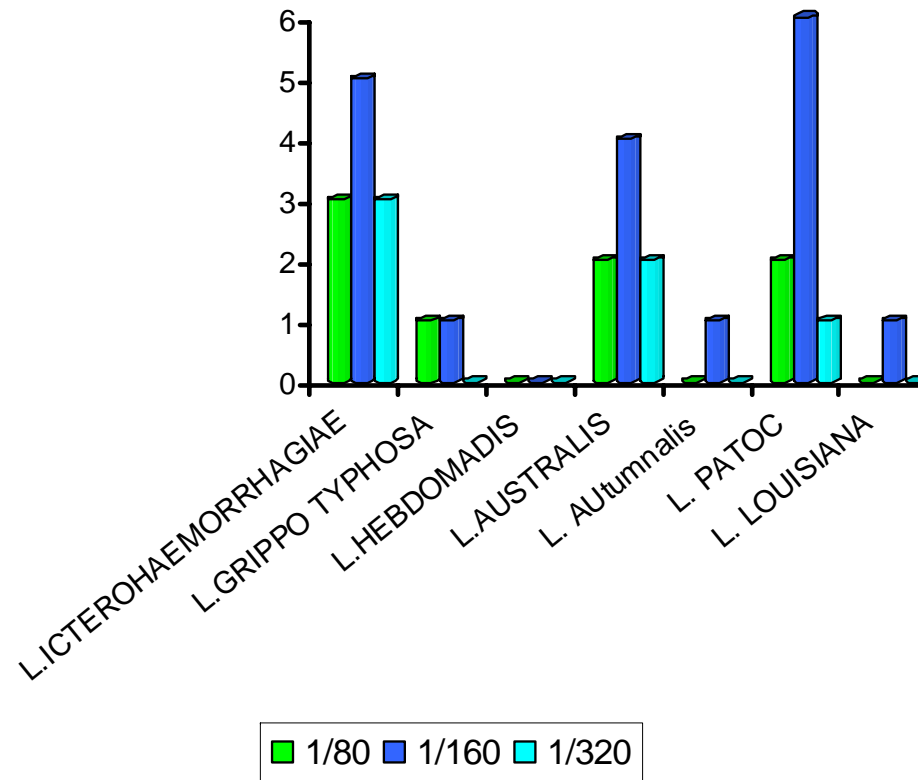




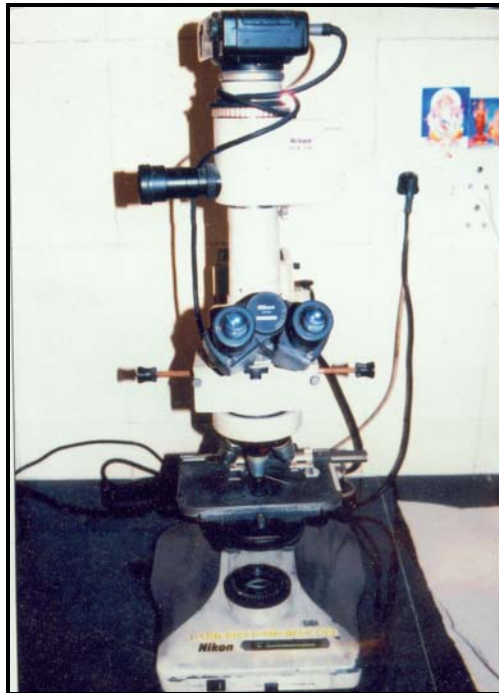
## MAT RESULTS DURING EARLY (3-5DAYS) & LATE (9-12 DAYS) FEBRILE PERIOD



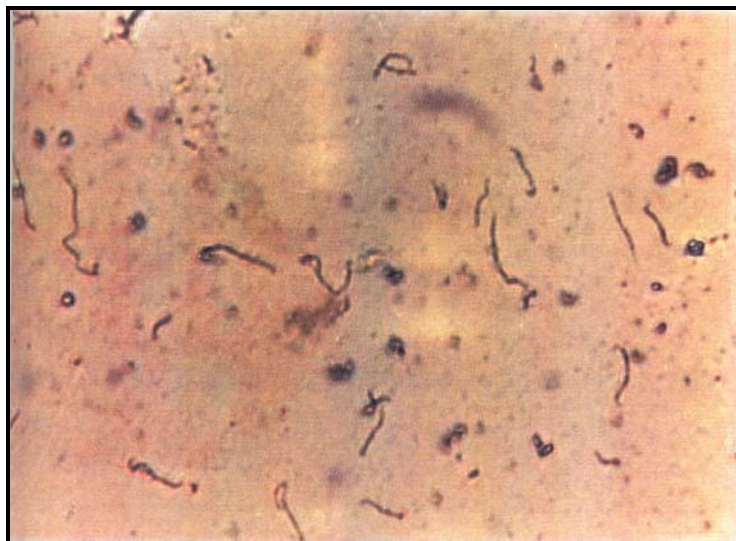
### MAT POSITIVE TITRE IN VARIOUS SEROVARS (LATE FEBRILE PERIOD)



*Dark Field Microscope*



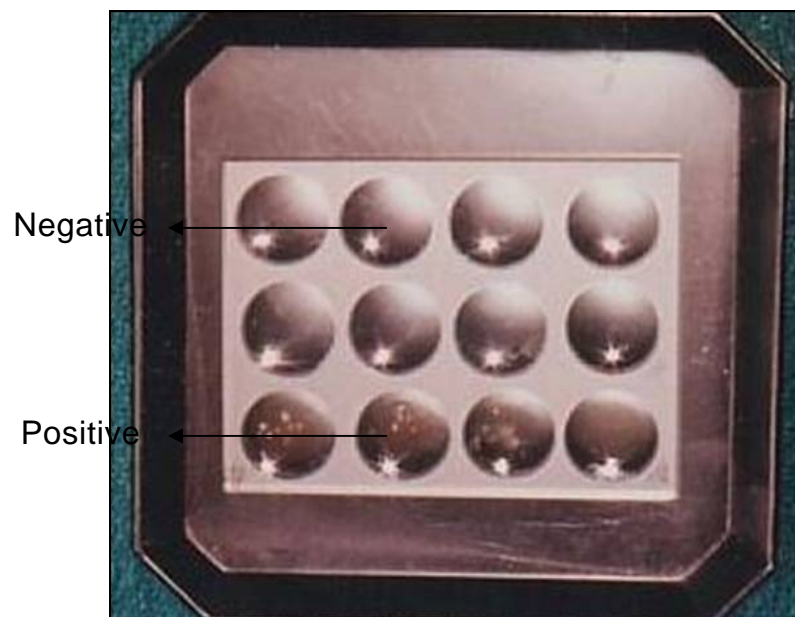
*Leptospira Fontana staining*



### *MSAT Setup*



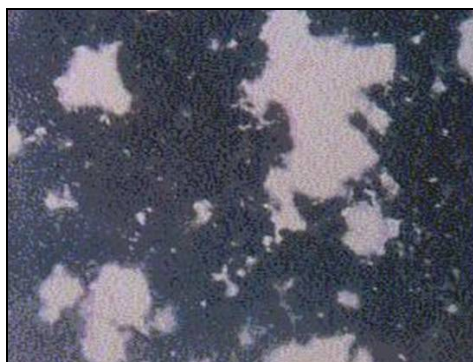
### *MSAT (Positive & Negative)*



## *MAT Setup*



*Positive*



*Negative*



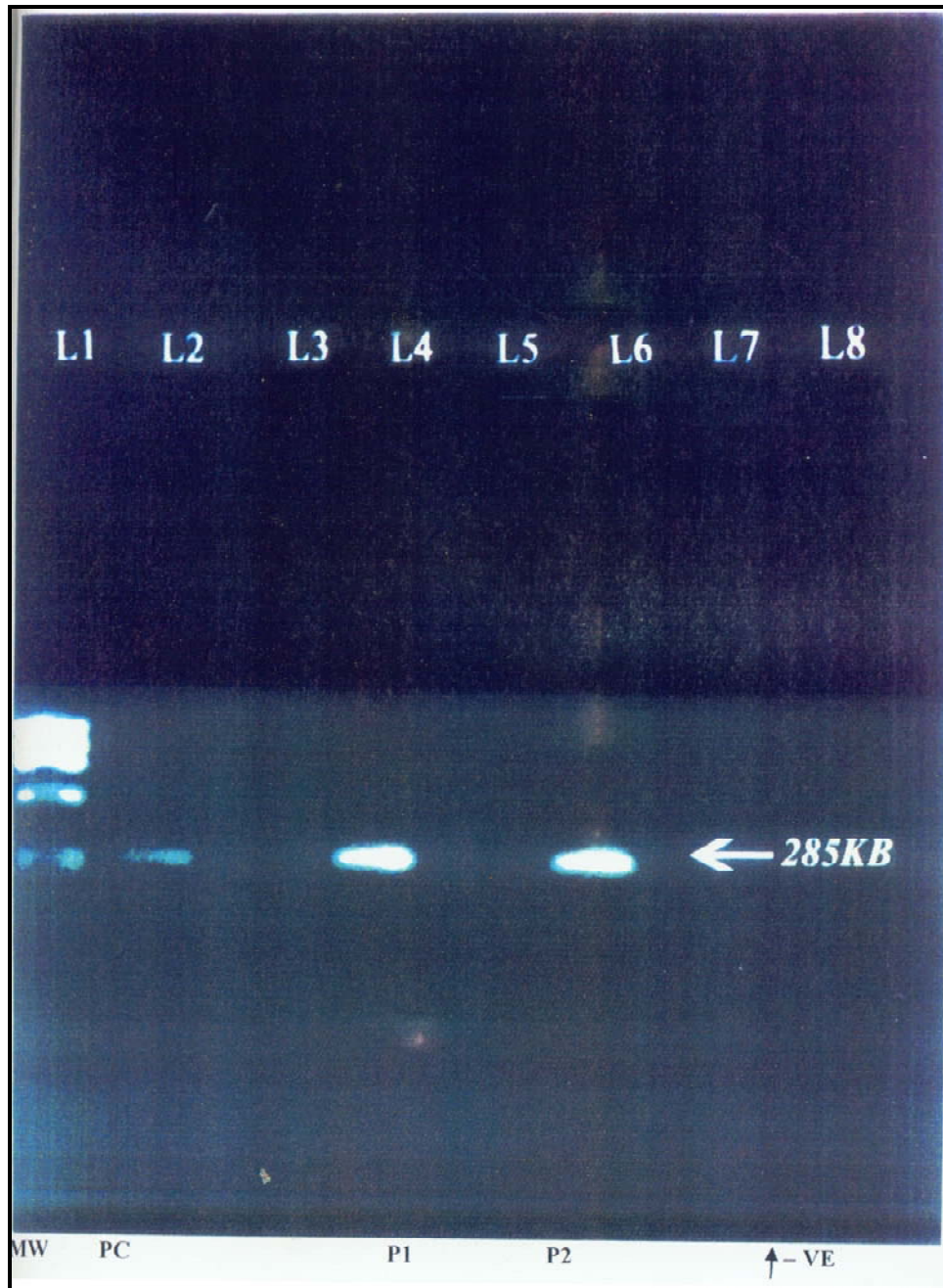
*MJ Thermal Cycler*



*Electrophoresis Apparatus*







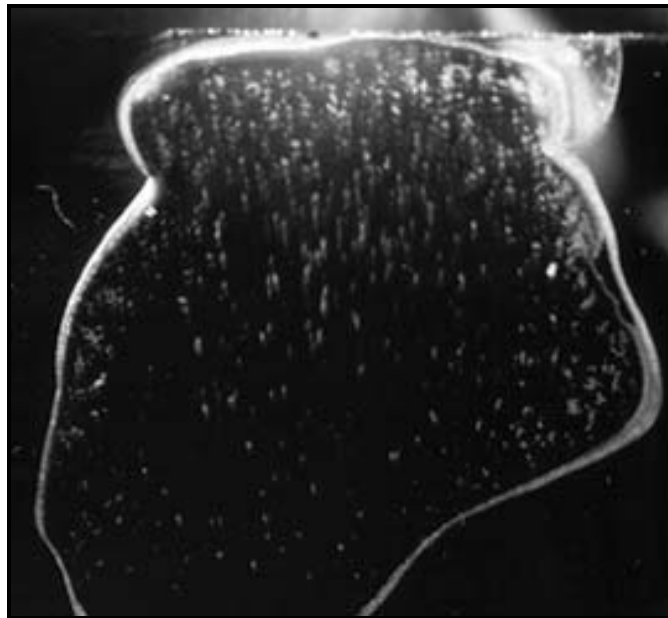
*MW - Molecular wt. Marker*

*P1 & P2 - Positive*

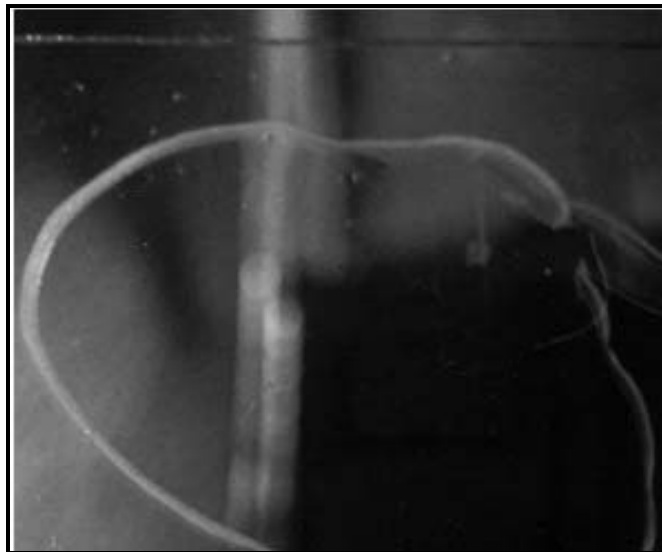
*PC - Positive Controller*

**PCR PRODUCTS GEL ELECTROPHORESIS**

### *Co Agglutination Test*



*Positive (Clearing of Suspension)*



*Negative (Smooth Suspension)*



## **DISCUSSION**

Leptospirosis is found to be an emerging infectious disease and has gained extreme public health importance in endemic areas of our country.

Its high prevalence in Chennai makes it necessary to devise a early diagnostic method. Early diagnosis is important because it helps in preventing the spread of active infection and early initiation of treatment.

This study was done with 100 serum samples from patients with symptoms suggestive of leptospirosis. The age distribution showed higher incidence in the age group of 21-40 yrs (42%) followed by 0-20 age group (26%) and >40 age group (32%) Mumford C.J. et. al. 1989<sup>42</sup> Marotto et al 1997 reported that common age group affected was between 12-24 yrs.

It is also interesting that the positive cases showed sex distribution ratio. male : female ( 58:42).

The predominant symptoms with which the patients presented were fever 100%, myalgia 55%, headache 40%, conjunctival suffusion 15%, Icterus 5%, etc. Pradeep C.S. et al 1995 in Chennai studied 253 cases of leptospirosis and showed that fever, headache and myalgia were the most commonly observed symptoms in the study group (91%).

In a study conducted by Joshi et al (2002)<sup>32</sup> in 200 patients of leptospirosis fever was the most common symptom (100%)

followed by headache (92%), myalgia (68%), conjunctival suffusion 35% & oliguria. 28%.

In humans, antileptospiral antibodies are detectable at about the 5-6 days of illness. As a result of immune response, leptospire are cleared from the blood after approximately 10 days of illness. Hence PCR is used as a diagnostic modality in the early period of illness. *Staphylococcus aureus* co-agglutination method has been used to detect the antigen in patient's serum for many infections caused by *Pneumococci*, *Hemophilus influenzae* typeb, *Vibrio cholera* (O<sub>139</sub>) *Salmonella typhi* & virus Infections due to *rota virus*, fungi like *Cryptococcus neoformans* & parasites like *Echinococcus granulosus*.

It is the objective of this study to assess the utility of Staphylococcal cells armed with anti-leptospiral antibody to detect in a rapid and perceptible manner the genus specific determinants on Leptospiral species The Co-agglutination reagent was used to screen 100 samples for the presence of leptospiral antigen by a simple slide agglutination Test. The results of this test were collaborated with polymerase chain reaction (PCR) which is considered as the gold standard antigen diagnostic test for leptospirosis.

In this study 100 samples were subjected to MSAT (Macroscopic Slide Agglutination Test), MAT (Microscopic Agglutination Test).Dark field microscopy, PCR and Co-agglutination test. The blood samples were collected during the early febrile period and repeat samples were collected during the follow up period. Macroscopic slide agglutination test &

Microscopic agglutination tests alone were done test in the repeat samples collected during the follow up period.

One of the widely accepted screening tests for leptospirosis - MSAT picked out 12 cases (33%) out of 100 in the early period and during 9-12 days of illness it picked up 34 cases (94%). Hence it is found that its sensitivity increases after a week time (Tab-8). Galton et al (1958)<sup>23</sup> found that patoc strain with killed antigen used for MSAT was more sensitive during the second week of illness. Sumathi et al 1996<sup>53</sup> found that the MSAT was positive in 39.8% when investigated in 1461 samples during the second week of illness.

Microscopic agglutination test, the gold standard test was performed on all the 100 cases. In early febrile period 8 cases (22%) were positive & in samples obtained during the late febrile period 32 cases (88%) were positive. MAT showed that predominant serovar to be *icterohaemorrhagiae* (37.5%) followed by *australis* (25%), *patoc* (25%), & *autumnalis* (12.5%). Pradeep et al 1995<sup>37</sup> reported that *autumnalis* (48.3%) was the predominant serovar isolated followed by *icterohaemorrhagiae* (31.1%).

In the early febrile period, Polymerase Chain Reaction was positive in 36 out of 100 cases when compared to dark field microscopy which was positive in 10 cases out of 100. Ramdass et al (1997)<sup>43</sup> compared PCR and DFM in 32 cases and found that PCR was more sensitive than DFM (PCR 21 cases positive, DFM 12 cases positive).

In a study by Merion et al (1992)<sup>41</sup> different samples like blood, CSF & urine were screened for the presence of leptospira by

PCR & cultural methods. It was found that PCR was positive in 4 out of 6 samples, & cultural methods detected only one positive sample. PCR could detect as few as 10 (Ten) leptospira present in the sample & was also free from false positivity due to cross reactions. Hence he concluded that PCR was highly sensitive & also faster than the cultural methods for detecting leptospira.

It is clear that PCR stands out as the best diagnostic modality during the early period of illness in leptospirosis. So it was decided to develop Staphylococcus aureus Co-agglutination test for the early detection of leptospirosis and evaluate it against PCR to test the efficacy of the performance, sensitivity & specificity results etc. Efforts have also been made to evaluate this test as a diagnostic tool for common laboratory testing.

In this study Co-agglutination test was positive in 30 cases out of 100 samples while DFM was positive in 10 cases out of 100 during the early period of illness. MSAT (macroscopic slide agglutination test) which is a widely accepted screening test was positive in only 12 cases out of 100 during the early febrile period. MAT (microscopic agglutination test), the gold standard test and the widely recommended test was positive only in 8 cases out of 100 during the early febrile period. This shows that co-agglutination is far more effective than DFM, MSAT & MAT for the diagnosis of leptospirosis during the early febrile period (3-5 days of illness).

PCR (polymerase chain reaction) was positive in 36 cases out of 100 cases. Considering PCR as the gold standard test for leptospirosis, the sensitivity and specificity of co-agglutination test in our study was 85.7% and 94.1% respectively. The positive likelihood ratio for co-agglutination test was 9 and negative

likelihood ratio was about 1 (0.93). The positive DLR and negative DLR values prove that this test stands better than many other tests in vogue. The following are the advantages of co-agglutination test:

- This test is rapid and enables easy detection within minutes when compared to MAT, MSAT & even PCR.
- It helps in earlier diagnosis of the disease before the progression of severe clinical symptoms.
- It aids in the detection of antigen unlike MAT & MSAT which detect only the antibodies.
- It avoids the usage of DFM thereby enabling any common laboratory to diagnose this disease.
- This is an inexpensive technique and can be used in field studies where large no. of cases have to be screened and where expensive microscopic units cannot be accessed.
- Can be used to easily detect the illness after natural calamities or disasters.
- Results obtained by this technique can be determined with unaided eyes.
- Many of the constraints of PCR like expensive equipment, need for trained hands, limited availability in developing countries are not seen in this method.

## **SUMMARY**

- Totally 100 clinically suspected cases of leptospirosis were included in the study.
- DFM, PCR, Co-agglutination, MSAT & MAT tests were done during the early febrile period (3-5 days) & MAT, MSAT were done during the late febrile period (9-12 Days)
- Fever was the most common presenting symptoms (100%) followed by myalgia (55%), headache (40%), conjunctival suffusion (15%), icterus (5%).
- Incidence was higher in the 21-40 age group.
- There was a preponderance in males (58%).
- Out of 100 samples tested during the early febrile period (3-5 days) 36 were positive by PCR, 30 by Co-agglutination, 12 by MSAT, 10 by DFM and 8 by MAT.
- Secondary screening of these cases during late febrile period by MSAT showed that 34 cases were positive. Majority of them showed 2+ agglutination titre (20).
- Secondary screening by MAT showed that 32 cases were positive and majority of them showed agglutination at 1/80 dilution of the serum (14). Ten (10) cases were positive in 1/160 dilution and 8 cases in 1/320 dilution of the serum.

- *Icterohaemorrhagiae* (37.5%) & *patoc* (37.5%) were the predominant serovars isolated during the early febrile period followed by *australis* (25%).
- Secondary screening in the late febrile period (9-12 days) showed that *icterohaemorrhagiae* was the most common serovar (34.5%) followed by *patoc* (28%) *australis* (25%) *grippotyphosa* (6.25%) *autumnalis* (3.12%) and *louisiana* (3.12%)

## **CONCLUSION**

Thus the comparative analysis with PCR and the various evaluation criteria shows that *Staphylococcal aureus* Co-agglutination method can be used as a screening test in the routine laboratory testing of leptospirosis. Further this test is simple, rapid, inexpensive and can be used in field settings and small unequipped laboratories located in remote areas of endemic regions. This genus specific test will help in initiating early treatment thus helping in reducing the morbidity & mortality due to leptospirosis.



## **APPENDIX – I**

### ***EMJH medium***

(Ellinughausen - Mccullough – Johnson – Harris medium)  
composition of EMJH medium base (Johnson Harris 1967)

### ***Ingredients***

Sodium Sulphate dibasic	1 gms
Potassium phosphate monobasic	0.3 gms
Sodium chloride	1 gms
Ammonium chloride	0.25 gms
Thiamine hydroxide	0.005gms

### ***EMJH Semisolid Medium***

For the preparation of 1000ml of semisolid medium 2.52 gms of EMJH base, 0.5ml of Tween 80 – and 1 gm of Agarose was added to 700ml of double distilled water. The pH of the medium was adjusted to 7.5 with 1N sodium Hydroxide and autoclaved at 15lbs for 15 mts at 121<sup>0</sup>C. After sterilisation the EMJH enrichment was added to EMJH base at a concentration of 10%

### ***EMJH liquid medium***

Liquid EMJH medium is prepared by dissolving 2.3 gms of EMJH base in 900ml of triple distilled water. The pH is adjusted to 7.5 After autoclaving at 15 lbs for 15 mts, the medium is added

aseptically with enrichment at 10% level. 100ml of enrichment is added with 900ml of EMJH medium.

### *EMJH Enrichment Medium*

The enrichment media is added to a final concentration of 10% to EMJH base to prepare the complete medium for the maintenance of leprospires.

### **MUELLER HINTON AGAR (HIMEDIA LABORATORIES MUMBAI)**

<i>Ingredients</i>	<i>gms/lt</i>
Beef Infusion	300
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17
Distilled water	1000
pH	7.3

100 ml of media was prepared and sterilised by autoclaving at 121<sup>0</sup>C for 15mts at 15 lbs pressure. After cooling, about 3ml of the media was transferred into sterile test tubes aseptically and made into slants.

## APPENDIX-II

### REAGENTS

#### 1. *Phosphate Buffered Saline (PBS).*

Ingredients	:	g/l
Sodium chloride	:	8.0
Potassium chloride	:	0.2
Disodium hydrogen phosphate	:	1.15
Potassium dihydrogen phosphate	:	0.2
Distilled water	:	1000 ml

pH was adjusted to 7.4.

The above ingredients were dissolved in sterile distilled water, and then filtered using filter paper (Whatmann No: 1)

#### 2.) *Phosphate Buffered Saline (pH:8.4).*

PBS was prepared as mentioned above and the pH was adjusted to 8.4 using 1N Sodium hydroxide solution.

#### 3.) *PBS + 1 % Sodium azide.*

PBS (pH: 7.4) was prepared and to 50 ml of this, 1 g of Sodium azide was added. The solution was made upto a final concentration of 100 ml.

#### 4.) *Saturated sodium sulfate solution*

The following concentrations of sodium sulfate were prepared:

- **36 % Sodium sulfate solution.**

3.6 g of Sodium sulfate was dissolved in 5 ml of sterile distilled water. The solution was then made up to 10 ml.

- **24 % Sodium sulfate solution.**

2.4g of Sodium sulfate solution was dissolved in 5 ml of sterile distilled water. The solution was then made up to 10 ml.

- **18 % Sodium sulfate solution.**

1.8g of Sodium sulfate solution was dissolved in 5 ml of sterile distilled water. The solution was then made up to 10 ml.

- **12 % Sodium sulfate solution.**

1.2g of Sodium sulfate solution was dissolved in 5 ml of sterile distilled water. The solution was then made up to 10 ml.

The sodium sulfate reagents prepared were then filtered using filter paper (Whatmann No: 1).

#### 5.) **0.05 % Sodium azide.**

0.05 g of Sodium azide was dissolved in 50 ml of sterile distilled water. The solution was then made up to 100 ml and then filtered using filter paper (Whatmann No: 1)

#### 6.) **1.5 % Formaldehyde.**

#### 7.) **Formalin 0.5 %**

## **APPENDIX - III**

### *Preparation of Antigens for Macroscopic Slide Agglutination Test*

1. Leptospires are grown in EMJH medium. Tubes with 3 ml medium are inoculated with a few drops of a 5-7 day old Leptospires culture. The inoculated tubes are incubated at 30° C for 5-7 days. After incubation, the cultures are examined by darkfield microscopy. If they are satisfactory (abundant growth and free from contamination) the cultures are dispensed into Ehrlenmeyer flask containing 50 ml EMJH medium and are then incubated for 5-7 days at 30<sup>0</sup> C in a shaking incubator. After incubation and microscopic examination, the cultures are dispensed in 500 ml of EMJH medium into a 1 litre flask and incubated again for 5-7 days at 30 °C in a shaking incubator. Then the cultures are examined by dark field microscopy. If they are satisfactory, formalin is added to a final concentration of 0.5% and the cultures are allowed to stand for atleast 30 minutes.
2. The cultures are centrifuged for 30 minutes at 10000 rpm to pack the leptospires. A rotation speed of less than 10000 rpm can also be used (e.g. 4000-5000 rpm for 1 hour or more).
3. The supernatant is discarded carefully and the tubes are allowed to drain in a slanted position for 2 hours.

4. The sediments in each tube are resuspended in 1.5 ml of a solution containing 0.5% formalin, 12% NaCl and 20% glycerol in distilled water added carefully. Addition of glycerol to the slide antigen prevents rapid drying during the test and has been found to protect the stability of the antigen. Hypertonic NaCl is selected because it has surface tension, which prevents antigen serum mixture from spreading too much during rotation of the glass test plate.
5. Pool the suspension in a 100 ml flask. Add glass beads and shake vigorously.
6. The antigens are kept at 4<sup>0</sup>C for two weeks before standardisation.

## PROFORMA

Name                      Age                      Sex                      Occupation

IP. No. :                      Address :

Date of Collection of 1<sup>st</sup> Sample :

Date of Collection of 2<sup>nd</sup> Sample :

Presenting Symptoms with duration :

H/o. Presenting illness :

Treatment given :

Investigations :

Microbiological profile :

- DFM for leptospira
- Co - agglutination for leptospira
- PCR - for leptospira
- MSAT
- MAT

Bio Chemical Profile :

- LFT
- RFT

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